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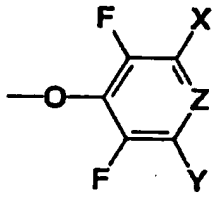
(57) Abstract

An analogue of a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound, wherein said analogue comprises a di- or tri-phosphate mimic at the site of phosphorylation, said mimic being a moiety of formula (I) wherein X and Y, which may be the same or different, are fluorine or hydroxy, and Z represents an electron with drawing group or a pharmacologically acceptable salt thereof, are useful for treating a disease which is caused or exacerbated by a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound.



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PHOSPHATE MIMICS

The present invention relates to a class of chemical substituents which mimic the effect of di- or tri- phosphate groups.

The regulation of many events related to growth and differentiation of cells is achieved by phosphorylation or dephosphorylation of biologically active compounds.

For example, the activation of *ras* oncogenes has been implicated in the onset of neoplasia (Kumar et al (1990) Science 248, 1101). The action of *ras* oncogenes is mediated through p21 protein products. These proteins have intrinsic GTPase activity and decreased activity associated with mutant forms of p21 is implicated in the transforming process (Lacal et al (1986) Cell 44 609). The crystal structure of the Ha-ras p21 protein in the GTP bound conformation has been determined (Pai et al (1989) Nature 341 209) and the X-ray structures of the active GTP-bound and the inactive GDP-bound forms have been compared (Schlichting *et al*, 1990, Nature, 345; 309).

These studies afford information which could ultimately prove useful in the design of inhibitors of the activation process acting via selective inhibition of the cycling between the GDP and GTP-bound forms of the mutant and overexpressed normal p21 proteins. Such agents might be analogues of GDP or GTP designed to displace the natural substrate from its binding site.

As a first step towards the design of analogues which could be therapeutically effective, the problem of transport into cells needs to be addressed. This is a problem not only in the present context, but in the design of inhibitors of nucleotide biosynthetic pathways which are the targets for clinically important antimetabolites such as 6-mercaptopurine and 5-fluorouracil which are converted intracellularly into their biologically active nucleotides. These nucleotides

cannot be used directly as drugs since they do not cross cell membranes. One strategy designed to overcome this is the use of pro-drug forms of the nucleotide which will liberate it intracellularly. Thus the lipophilic pivaloyloxymethyl ester of 5-fluorodeoxyuridylic acid can revert to the free acid by enzymatic hydrolysis (Farquhar (1983) J. Pharm. Sci 72 324). A variation on this theme is the synthesis of cyclic phosphotriesters, and phosphoramidates derived from 2'-deoxy-5-fluorouridine (Hunston et al (1984) J. Med. Chem. 27 440, Farquhar et al (1983) J. Med. Chem. 26 1153 and Jones et al (1984) J. Chem. Soc. Perkin TransI, 1471). An alternative approach is the use of nucleotide pro-drugs or analogues which, although retaining a charge, are more readily transported than nucleotides. An example is bis(6-mercaptopurine-9-β-D-ribofuranoside)-5'-5'-monophosphate (Johnston et al (1985) Brit. J. Cancer 51 505) as a potential pro-drug for the active nucleotide metabolite of 6-mercaptopurine.

A different approach to the problem of transport dispenses with phosphate functionalities with their associated intrinsic transport problems and replaces them with alternative functionalities designed to mimic as far as possible their spatial arrangements and electronic properties. For the present main goals, those of competitive inhibition of GTP or GDP binding to Ras p21 and of binding of farnesyl pyrophosphate to Ras protein farnesyl transferase, mimicry of di- or triphosphate as opposed to monophosphate residues is required. Binding of Mg^{2+} is generally important to the functions of naturally-occurring nucleoside di- and triphosphates and this is an additional consideration in the design of diphosphate or triphosphate mimics. Taking this into consideration, a branched furan-2-acetic amide derivative of adenosine has been designed as an ATP mimic (Otoski et al (1988) Tetrahedron Lett. 29 2615).

A further cofactor involved in the regulation of the Ras protein is the co-substrate farnesyl pyrophosphate. This compound provides a farnesyl group for the Ras protein,

enabling the Ras protein to lodge in the membrane of the cell. The attachment of the farnesyl group to the Ras protein releases the pyrophosphate group.

Other related examples of biologically active molecules containing the di- or triphosphate residue which associate with, or act upon, proteins include the other three deoxyribonucleoside triphosphates (ATP, CTP, TTP) which together with GTP are substrates for nucleoside triphosphate polymerases which assemble DNA. Other isoprenyl pyrophosphates besides farnesyl pyrophosphate include isopentenyl, geranyl and geranylgeranyl pyrophosphates. The first two, and farnesyl pyrophosphate, are intermediates in the biosynthesis of cholesterol whereas geranylgeranyl pyrophosphate, has a regulatory role in protein prenylation comparable to that of farnesylation of Ras protein.

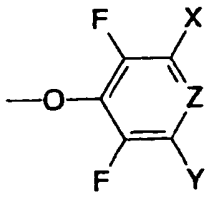
We have surprisingly found, through studies of molecular models, that certain perfluoroaryl groups and related moieties comprise an overall spatial and electronic distribution which mimic those found in di- and tri-phosphate groups.

Our initial studies have focused upon using such moieties to provide analogues of GTP and GDP and we have found that one such analogue competes for the nucleotide binding site of the truncated (1-166) mutant leu-61 Ha-ras. We have extended this work into the production of analogues of farnesyl pyrophosphate and our initial findings indicate that these analogues have activity as inhibitors of ras protein farnesyl transferase.

These results together with our modelling studies indicate that the phosphate mimics we have studied have widespread application as phosphate mimics in a variety of biological environments.

Accordingly, the present invention provides an analogue of a biologically active compound whose activity within a cell is

regulated by phosphorylation of said compound, wherein said analogue comprises a di- or tri- phosphate mimic at the site of phosphorylation, said mimic being a moiety of the formula (I):



(I)

wherein X and Y, which may be the same or different, are fluorine or hydroxy, and Z represents an electron withdrawing group or a pharmacologically acceptable salt thereof.

Preferably, X and Y are both fluoro, and more preferably X is hydroxy and Y is fluoro. However, it is possible for both X and Y to be hydroxy. Z is preferably =N or =C(R)- and R is preferably carboxy or a mimic thereof (for example 5-tetrazolyl), trifluoromethyl, nitro, cyano, alkyl- or aryl-sulphonyl ($-SO_2R$), a sulphonic acid ($-SO_3H$) or ester thereof or methylene phosphonic acid ($-CH_2PO_3^{2-}$) or ester thereof. When X is hydroxy and Y is fluoro, R is preferably carboxy or nitro.

Preferred analogues according to the invention are also those wherein Z is =N- or $=C(NO_2)-$.

As indicated above, the biologically active compound is preferably GTP or GDP, or a farnesyl pyrophosphate. However, the compound may also be another of the naturally occurring ribonucleoside or deoxyribonucleoside di-or triphosphates, synthetic nucleoside or acyclonucleoside di- or triphosphates which are the active metabolic products of nucleoside precursors having in particular anticancer or antiviral activity, other naturally occurring prenyl pyrophosphates particularly isopentenyl, geranyl and geranylgeranyl

pyrophosphates and synthetic analogues of these as later defined.

Examples of compounds of the invention include:

5'-O-(2,3,5-trifluoro-6-hydroxy-4-pyridyl)guanosine;
5'-O-(2,3,5,6-tetrafluoro-4-pyridyl)guanosine;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)guanosine;
5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)guanosine;
5'-O-(3,5-difluoro-2,6-dihydroxy-4-pyridyl)guanosine;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-cyano)phenylguanosine;
5'-O-(2,3,5,6-tetrafluoro-4-carboxy)phenylguanosine;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]pyridine;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]cyanobenzene;
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]pyridine;
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene; and
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene sodium salt;
2,3,5-trifluoro-6-hydroxy-4-(trans, trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy) benzoic acid, sodium salt;
2,3,5,6-tetrafluoro-4-(trans,trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy) benzoic acid, sodium salt;
1-[(E)-3,7-dimethyl-2,6-octadien-1-yloxy]-2,3,5,6-tetrafluoro-4-nitrobenzene;
1-[(E)-3,7-dimethyl-2,6-octadien-1-yloxy]-2,3,6-trifluoro-5-hydroxy-4-nitrobenzene;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)-2'-deoxyuridine;
5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine; and
4-(2'deoxyuridin-5'-yloxy)-2,3,5-trifluoro-6-hydroxybenzoic acid.

In a further aspect, the invention provides a pharmaceutical composition comprising an analogue of a biologically active compound according to the invention together with a pharmaceutically acceptable carrier or diluent.

Analogues of a biologically active compound according to the invention may be used in a method of treatment or therapy of the human or animal body, for example in the treatment of cancers associated with aberrant expression of the Ras protein or mutated forms thereof. Thus the invention provides a method of treating a disease of the human or animal body in which said disease is caused or exacerbated by a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound which method comprises administering to a patient suffering from said disease an effective amount of a compound according to the invention.

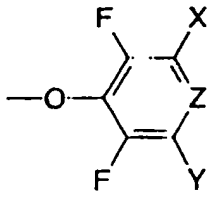
Cancers associated with aberrant expression of the ras protein include human colon, mammary and pancreatic tumours. Mutations in Ras proteins are found in a significant number of human cancers including approximately 50% of colon and 90% of pancreatic carcinomas.

Other diseases which may be treated include hypercholesterolemia (Ciosek et al (1993) The Journal of Biological Chemistry 268 (33) 24832-24837), viral diseases such as herpes, HIV and cytomegalovirus retinitis, gout, some parasitic diseases, and glomerular (kidney) disease.

The analogues of the invention are also useful in examining the role of phosphorylation in the cell cycle and in cell signalling pathways by using the analogues in *in vitro* experiments designed to study the role of phosphorylation on a particular biological compound or pathway. This may be accomplished by adding the appropriate phosphate analogue compound of the invention to a culture of cells in which the target pathway is active, and measuring the effect of said analogue on said cell culture. For example, the cell culture

may be tumour cells which are taken from a patient with a tumour caused by a mutation in the Ras protein.

In a further aspect, the invention provides a chemical moiety of the formula (I):



(I)

wherein X, Y and Z are as defined above for use as a di- or tri- phosphate mimic. In an additional aspect, the invention also provides the use of a chemical moiety of the formula (I) as defined above as a di- or tri- phosphate mimic.

Detailed description of the invention.

The biologically active compound may be any naturally occurring or synthetic compound whose biological activity within a cell is regulated by phosphorylation. Such compounds include nucleotides (e.g nucleotide di- tri- phosphate of adenosine, guanosine, cytosine, thymidine, uridine or other naturally occurring or synthetic nucleosides such as AZT, ddI or ddC and acyclonucleosides such as acyclovir and gancyclovir), and co-factors and substrates of enzymes such as purine nucleoside phophorylase, viral DNA polymerase, protein tyrosine kinases, MAP kinase, dUTPase and squalene synthase.

Preferably, the cell containing the biologically active compound will be a mammalian cell, particularly a human cell. However many biologically active molecules are evolutionarily conserved and are found throughout eukaryotic and prokaryotic cells, although the regulation and targets of such molecules may differ. Where the phosphate mimic moieties of the invention are used in the study of phosphate groups in

biological pathways, this may include prokaryotic pathways as well as those found in eukaryotes, including yeast.

When the biologically active compound is a farnesyl pyrophosphate, it is preferred that the farnesol moiety of such a compound is the trans,trans-farnesol moiety. However, since the farnesyl group is a trimer of isoprenyl groups other steric configurations of the trimer may be used. Synthetic analogues of such farnesyl compounds may also be used, e.g analogues which have been substituted by one or more fluoro, chloro, bromo, iodo, methyl or trifluoromethyl groups. A fluorine substitution is likely to enhance the metabolic stability of a farnesyl pyrophosphate analogue.

The farnesyl moiety may also be replaced by a shorter or longer unsaturated alkyl group or substituted derivatives as defined above. A suitable shorter group is geranyl which may have an advantage in terms of stability compared with the corresponding farnesyl compound.

Any reference to any of the above analogues of the invention also includes a reference to a physiologically acceptable salt thereof. Salts according to the invention which may be conveniently used include physiologically acceptable base salts, eg derived from an appropriate base, such as alkali metal (e.g. sodium), alkaline earth metal (e.g. calcium and magnesium) salts and ammonium salts. Physiologically acceptable acid addition salts, including the hydrochloride and acetate salts may also be made where appropriate. Prodrugs such as biologically degradeable esters (eg. acetate esters) may also be used.

The analogues according to the invention may be administered to mammals including humans by any route appropriate to the condition to be treated, suitable routes including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and

epidural). It will be appreciated that the preferred route may vary with, for example, the condition of the recipient.

The amount required of the individual analogue will depend upon a number of factors including the nature of the analogue, the severity of the condition to be treated and the identity of the recipient and will ultimately be at the discretion of the attendant physician.

For example where nucleoside analogues such as analogues of GDP or GTP are provided, a suitable, effective dose will be in the range 0.05 to 250 mg per kilogram body weight of recipient per day, preferably in the range 0.5 to 20 mg per kilogram body weight per day. The desired dose may if desired be presented as two, three, four or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing 10 to 1000 mg, preferably 20 to 500 mg and most preferably 100 to 400 mg of active ingredient per unit dosage form.

While it is possible for the compounds to be administered alone it is preferable to present them as pharmaceutical formulations. The formulations of the present invention comprise at least one active ingredient, as above defined, together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients thereof.

The formulations include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active

ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

Pharmaceutical formulations for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, paste, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipient or diluents.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostatics and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water or hypotonic aqueous solutions such as aqueous dextrose or buffer, immediately prior to use.

The compounds according to the invention may be employed alone or in combination with other therapeutic agents for the treatment of the above infections or conditions. Combination therapies according to the present invention comprise the administration of at least one analogue of the invention or a physiologically functional derivative thereof and at least one other pharmaceutically active ingredient. The active ingredient(s) and pharmacologically active agents may be administered together or separately and, when administered separately this may occur simultaneously or sequentially in any order.

Reference may be made to the accompanying examples and the following description for methods for making the compounds of the invention.

In general, the phosphate groups which the moieties of the formula (I) mimic are covalently linked to a biologically active compound via a phosphate-ester bond. The unphosphorylated biologically active compound usually has a hydroxy group which is linked to the phosphate moiety. This hydroxy group may be used to create an ether bond between the biologically active compound and the moiety of formula (I).

Thus where the group Z is =N- pentafluoropyridine may be used under phase transfer catalysis in a two phase system consisting of an aqueous alkaline phase, an organic phase in the presence of a phase transfer catalyst such as a tetraalkylammonium salt by methods analogous to those described in M.Jarman and R.McCague, "Octafluorotoluene as a reagent for the selective protection of alcoholic and phenolic functions: synthesis and cleavage of perfluorotolyl and other perfluoroaryl ethers of steroids and other model compounds" J.Chem.Res. 1985,(S) 114-115; (M) 1301-1341.

This will provide analogues with moieties in which X and Y are both fluorine. These may be converted to hydroxy groups by treating the analogue with strong base (e.g sodium hydroxide at about 1.0 M) or using concentrated (e.g.50%w/w)

aqueous NaOH under conditions of phase transfer catalysis by methods analogous to those described in M.Jarman, S.E.Barrie, J.J.Deadman, J.Houghton, R.McCague and M.G.Rowlands "Hydroxyperfluoroazobenzenes: novel inhibitors of enzymes of androgen biosynthesis" J.Med.Chem. 1990, 33, 2452-2455.

Compounds in which Z is a group C(R) may in general be prepared in an analogous manner starting with the appropriate pentafluorobenzene derivative substituted with the group R, e.g. pentafluoronitrobenzene, etc.

Compounds in which X and Y are both hydroxy may be made by a method analogous to that described in the Jarman and McCague 1985 reference cited previously. Thus, appropriate treatment of a tetrafluorinated intermediate of the invention with sodium methoxide in dimethylformamide leads to successive replacement of fluorine flanking the substituent R by methoxyl. The use of an alkali metal alkoxide in which the alkyl group is a removable protecting group (e.g. benzyl, phenyl, tetrahydropyranyl) leads to intermediate di(alkoxy) derivatives capable of conversion into compounds wherein X and Y are both hydroxyl. The strategy is illustrated in example 10.

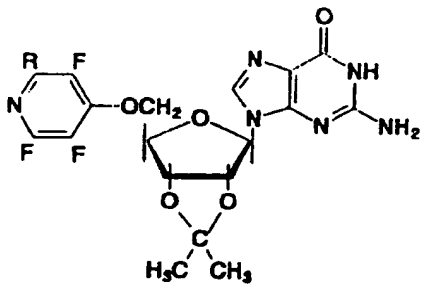
It may sometimes be necessary to protect the biological compound in modifying the phosphate moiety. In the nucleoside di- and triphosphate mimics described, the 2 and 3-hydroxyls on the sugar moiety are protected by the isopropylidene group which is removed with acid after insertion of the hydroxyl moiety.

The following examples illustrate the invention.

EXAMPLE 1

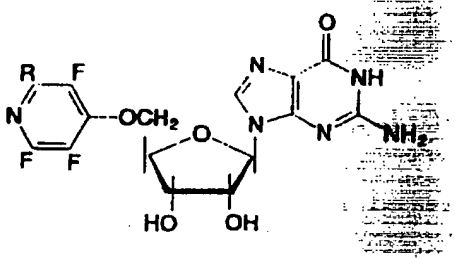
A protected perfluoropyridylguanosine (1) was conveniently prepared by the phase-transfer catalysed reaction of 2',3'-O-isopropylideneguanosine with pentafluoropyridine. No

reaction product involving nucleophilic sites on the guanine residue was detected under these conditions showing that the perfluoroaryl group is here acting as a hard electrophile, as in previously reported reactions of perfluoroarenes. The hydroxyl function was conveniently introduced by taking advantage of the solubility of guanosine derivatives in aqueous NaOH by virtue of the ionizable imide NH function. Acidic hydrolysis of the protected intermediate 2 thus produced afforded the target compound 3. Acidic hydrolysis of 1 itself produced the 5'-O-tetrafluoropyridylguanosine 4.



1: R = F

2: R = OH



3: R = OH

4: R = F

5'-O-(2,3,5,6-Tetrafluoro-4-pyridyl)-2',3'-O-isopropylideneguanosine (1).

A mixture of 2',3'-O-isopropylideneguanosine (3.23 g, 10 mmol), pentafluoropyridine (4.06 g, 2.63 ml, 24 mmol), tetra-n-butylammonium hydrogen sulphate (3.39 g, 10 mmol), 1M NaOH (50 ml) and CH_2Cl_2 (50 ml) was stirred for 2 h at room temperature, then 1 M H_2SO_4 (15 ml) was added. The organic phase was concentrated and the residue crystallised from EtOH (20 mL) to give 1 (3.29 g, 70%) as a white solid: mp 225°C decomp; δ_H (Me_2SO-d_6) 1.34, 1.54 (2s, each 3, $(CH_3)_2C$), 4.48 (m, 1, H-4'), 4.68 (dd, 1, H-3'), 4.82 (dd, 1,H-2'), 5.28 (m, 2, H-5'), 6.08 (d, 1, $J_{1',2'}$ = 1.25 Hz, H-1'), 6.57 (s, 2, NH_2), 7.81 (s, 1, H-8), 10.74 (s, 1, NH); δ_F -92.2 (A_2X_2, 2,F-2,6; -157.9 (A_2X_2, 2,F-3,5). Analysis found: C,45.69; H,3.45;

F,16.04; N,17.78. $C_{18}H_{16}F_4N_6O_3$ requires: C,45.77; H,3.41, N, 17.79; F,16.09%.

5'-O-(2,3,5-Trifluoro-6-hydroxy-4-pyridyl)-2',3'-O-isopropyl-ideneguanosine (2).

A solution of 1 (944 mg, 2 mmol) in 1 M NaOH (20 ml) was kept at 40°C for 16 h then 1 M H_2SO_4 (10 ml) was added. The resulting white solid was recovered by filtration and recrystallised from EtOH-H_2O, 1:1 to give 2 (671 mg, 69%) as colourless plates:

mp > 190°C (decomp); Anal found: C,44.45; H,3.97; F,11.46; N,17.20. $C_{18}H_{17}F_3N_6O_6.H_2O$ requires C,44.27; H,3.92; N,17.21; F,11.67%.

5'-O-(2,3,5-Trifluoro-6-hydroxy-4-pyridyl)guanosine (3).

A mixture of 2 (470 mg, 0.96 mmol) and 1 M H_2SO_4 (10 ml) was stirred first at 60°C for 10 min to effect solution, then at 40°C for 16 h, then 1 M NaOH (20 ml) was added to the cooled solution. After 3 h, 3 (326 mg, 76%) was recovered by filtration. It could be recrystallised from a large volume of water: mp >190°C (decomp); δ_H (Me_2SO-d_6) 4.15 (m, 1, H-4'), 4.24 (m, 1, H-3'), 4.47 (m, 1, H-2'), 4.62 (m, 2, H-5'), 5.38 (brd, 1,3'-OH), 5.59 (brd, 1,2'-OH), 5.76 (d, 1, $J_{1',2'}$ = 5.4 Hz, H-1'), 6.49 (s, 2, NH_2), 7.82 (s, 1, H-8), 10.32 (s, 1, NH); δ_F = 93.7 (app t, 1, F-6), -158.65 (d, 1, $J_{5,6}$ = 25.6 Hz, F-5), -167.5 (d, 1, $J_{3,6}$ = 25.7 Hz, F-3); m/z (FAB) 431 ([M+H]$^+$). Analysis found: C,40.54; H.3.46; F,12.71; N,19.02.$C_{15}H_{13}N_6O_6$0.5H_2O requires: C,41.01, H,3.21; F,12.97; N,19.13%.

5'-O-(2,3,5,6-Tetrafluoro-4-pyridyl) guanosine (4).

A solution of 1 (472 mg, 1 mmol) in trifluoroacetic acid (4 ml) was diluted with water (4 ml) and the solution set aside for 6 h, then concentrated to remove most of the trifluoroacetic acid. Solid $NaHCO_3$ was added to neutrality and the solid product was recovered by filtration and washed with H_2O to yield 4 (420 mg, 93%) which could be recrystallised from a large volume of H_2O: mp 216-219°C; δ_H (Me_2SO-d_6) 4.22 (m, 1, H-4'), 4.27 (dd, 1, H-3'), 4.52 (dd,

1, H-2'), 4.77 (m, 2, H-5'), 5.63 (d, J = 5.8 Hz, 3'-OH), 5.76 (d, J = 5.25 Hz, 2'-OH), 5.76 (d, $J_{1',2'}$ = 5.25 Hz, H-1'), 6.48 (s, 2, NH_2), 7.83 (s, 1, H-8), 10.64 (s, 1, NH). Analysis found: C,41.22; H,3.11; F,17.04; N,18.68. $C_{15}H_{12}F_4N_6O_5.0.5\ H_2O$ requires: C,40.82; H,2.97; F,17.22; N,19.05%.

EXAMPLE 2

5'-O-(2,3,6-Trifluoro-5-hydroxy-4-nitrophenyl)guanosine (7)

1. Synthetic Strategy

Following the successful synthesis of 3, it was proposed that 5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)guanosine may also be accessible using similar perfluoroarene chemistry. A protected perfluoronitrophenylguanosine 5 was prepared in a similar manner to 1, by a phase-transfer catalysed reaction of pentafluoronitrobenzene with 2',3'-O-isopropylideneguanosine. Again the hydroxyl function was introduced by nucleophilic substitution to give 6, which after acidic hydrolysis yielded 7.

Experimental

5'-O-(2,3,5,6-Tetrafluoro-4-nitrophenyl)-2',3'-O-isopropylideneguanosine (5).

A mixture of 2',3'-O-isopropylideneguanosine (3.232g, 10 mmol). pentafluoronitrobenzene (2.577g, 12 mmol), tetra-n-butylammonium hydrogen sulphate (3.395g, 10 mmol), CH_2Cl_2 (50 ml) and M NaOH (50 ml) was stirred for 0.5 hrs at room temperature, then a further 2.56g (12 mmol) was added and stirring continued. 2N H_2SO_4 (30ml) was added to the mixture. The organic phase was recovered and concentrated to a foam which was dissolved in warm isopropanol (30ml) and allowed to crystallise to give 2.180g of solid. Recrystallisation using EtOH (30ml) gave 5 (1.89g, 37% yield), as a yellow solid: mp. 165-168°C (decomp): δ_H = 1.34, 1.54 (2s, each 3, $(CH_3)_2C$), 4.2-4.6 (m, 2, H-5'), 4.6-4.8 (m, 1, H-4'), 5.20 (m, 1, H-3'), 5.33 (d, 1, H-2'), 6.06 (s, 1, H-1'), 6.49 (brs, 2, NH_2), 7.78 (s, 1, H-8), 10.64 (s, 1, NH), δ_F = -147.1 (A_2X_2, F-3,5), -154.8 (A_2X_2, F-2,6): Anal. found:

C, 44.30%; H, 3.13%; N, 16.18%; F, 14.86%. $C_{19}H_{16}N_6O_7$ requires: C, 44.20%; H, 3.12%; N. 16.28%; F, 14.72%.

5'-O-(2,3,6-Trifluoro-5-hydroxy-4-nitrophenyl)-2',3'-O-isopropylideneguanosine (6).

A mixture of 5 (516mg, 1 mmol) and M NaOH (10 ml) was stirred at room temperature for 2 hrs. The solution was then filtered and 2N H_2SO_4 (5ml) added. The resulting yellow solid was recovered by filtration and was washed with water, to give 6 (216mg, yield 79%): δ_H= 1.34, 1.54 (2s, each 3, $(CH_3)_2C$), 4.42-4.51 (2m, 3, H-4'+H-5'), 5.18 (dd, 1, H-3'), 5.29 (d, 1, H-2), 6.05 (s, 1, H-1'), 6.47 (brs, 2, NH_2), 7.81 (s, 1, H-8), 10.62 (s, 1, NH), δ_F = 151.8 (d, 1), -151.9 (s, 2): Anal. found: C, 43.59%; H, 3.39%; N, 15.79%; F, 10.44%. $C_{19}H_{17}N_6F_3O_8.0.5H_2O$. requires: 42.87%; H, 3.60%, N, 15.79%; F, 10.71%.

5'-O'-(2,3,6-Trifluoro-5-hydroxy-4-nitrophenyl)guanosine (7).

A solution of 6 (1.032g, 2 mmol) in M NaOH (20ml) was kept at 40°C for 16 hrs then 2N H_2SO_4 (10ml) was added. The resulting pale yellow solid was recovered by filtration and recrystallised from EtOH/H_2O 1:1, to give 7 (119mg, 50% yield) as colourless plates: mp, 193-195°C (decomp): δ_H= 4.18, 4.24 (2t, 2, H-5'), 4.50 (m, 3, H-2',3',4'), 5.3, 5.5 (2 brs, each 1, 2',3'-OH), 5.76 (d, 1, $J_{1',2'}$ = 5.5 Hz, H-1'), 6.43 (brs, 2, NH_2), 7.83 (s, H-8), 10.58 (s, 1, NH). δ_F = -151.4 (d, F-6), -151.8 (dd, F-3), -162.8 (d, F-2). Anal. found: C, 38.44%; H, 3.00%; N, 16.44%; F, 11.11%, $C_{16}H_{13}N_6F_3O_8.1.5H_2O$ requires: C, 38.33%; H, 3.72%, N, 16.76%, F, 11.37%.

EXAMPLE 3

2,3,5,6-Tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]pyridine

To a stirred solution of trans,trans farnesol (2.224 g, 10 mmol) in dichloromethane (20 ml) was added pentafluoropyridine (1.69 g, 10 mmol) and tetra-n-butylammonium hydrogen sulphate (0.34 g, 1 mmol) as a phase transfer catalyst then M NaOH (20 ml). After 1 hr, a further

10 mmol pentafluoropyridine was added with continued stirring. The product after a further hour was treated with 2N H_2SO_4 (10 ml) and the organic phase was separated and concentrated. The residue was portioned between light petroleum (bp 60-80°C) and water and the petrol layer was separated and concentrated to an oil. This should be distilled using an oil pump (Kugelrohr apparatus).

EXAMPLE 4

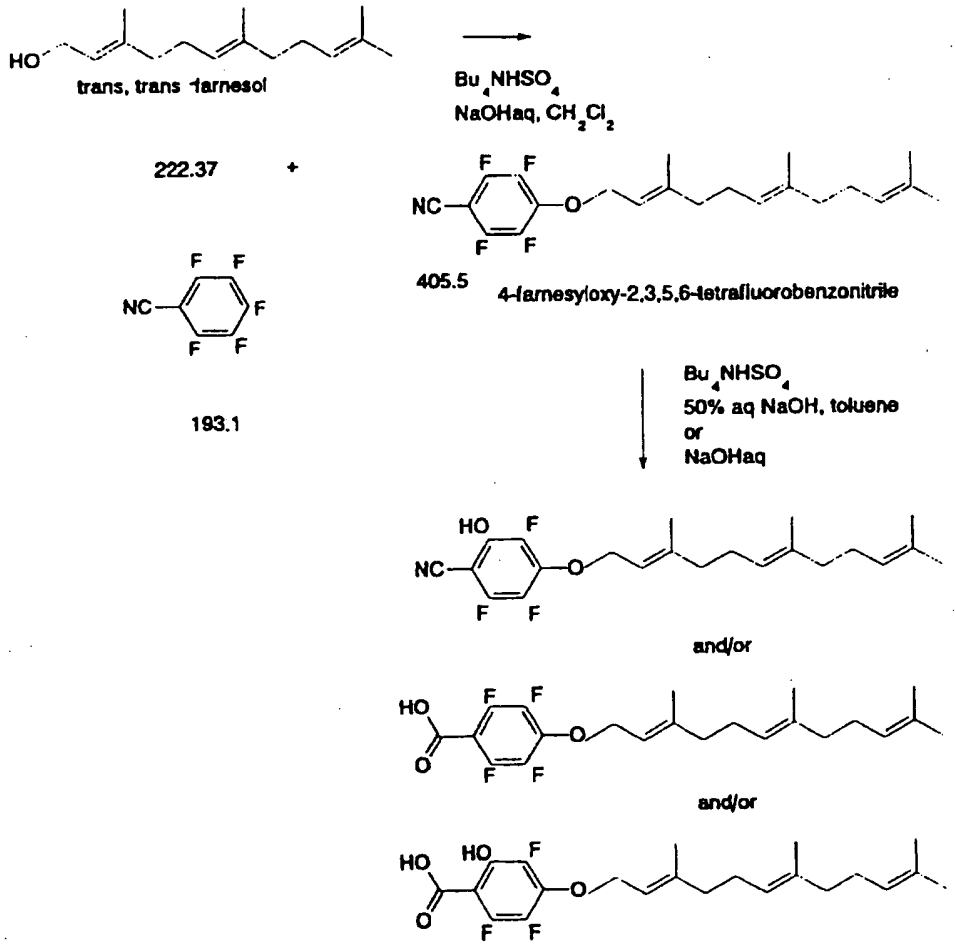
2,3,5,6-Tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene

To a solution of trans, trans-farnesol (1.112g, 5.0 mmol) in dichloromethane (10 ml) was added pentafluoronitrobenzene (1.065, 5.0 mmol) and tetra-n-butylammonium hydrogen sulphate (0.17g, 0.5 mmol) then 1M NaOH (10 ml). After 1 hr, a further 0.5g pentafluoronitrobenzene was added with continued stirring. The product after a further hour was treated with 1M H_2SO_4 (5 ml) and the organic phase was separated, dried over $MgSO_4$ and concentrated. Flash silica chromatography, eluting with hexane-dichloromethane (20:1), afforded the title compound as a yellow oil (1.16g, 56%); ^{1}H-NMR (250 MHz, $CDCl_3$) δ 1.59 (6H, s, CH_3), 1.68 (3H, d, J=0.8Hz, CH_3), 1.74 (3H, d, J = 0.8 Hz, CH_3), 2.01-1.96 (8H, m, CH_2), 4.92(2H, d, J = 7.3 Hz, OCH_2), 5.06 (2H, m, vinylic), 5.46 (1H, t, J = 7.3 Hz, vinylic OCH_2CH); ^{19}F-NMR (235 MHz, $CDCl_3$) δ-158.22(2F, m, meta to NO_2), -151.56 (2F, m, ortho to NO_2) AA'XX' system; HRMS Found: m/z 414.1695; $C_{21}H_{24}F_4NO_3$ requires 414.1692; found. C 60.93, H 6.29, N 3.24, F 17.37. $C_{21}H_{25}F_4NO_3$ requires: C 60.71, H 6.07, N 3.37, F 18.29.

EXAMPLE 5

2,3,5,6-Tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy] cyanobenzene

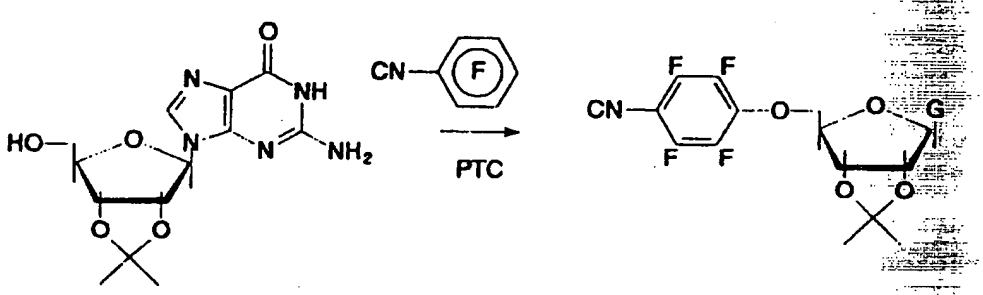
The preparation is carried out as above except that pentafluorobenzonitrile (2 x 1.93 g) is used. The following scheme illustrates the process used and processes that can be used to make corresponding 5-hydroxy and 4-carboxy substituted compounds.



An analogous attempt to prepare an ortho-hydroxybenzoic acid by this route in a guanosine analogue led directly to hydrolysis of the cyano to the carboxyl group without any isolation or detection of an intermediate cyanohydroxy derivative. This route and the characterization of products is described in Examples 6 to 9.

EXAMPLE 6

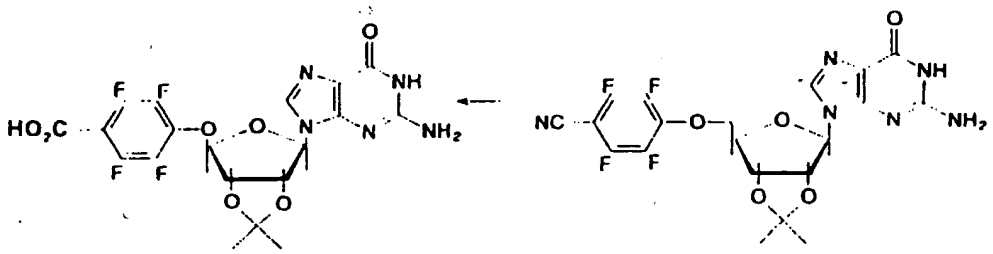
5'-O-(4-cyano-2,3,5,6-tetrafluoro)phenyl-2',3'-O-isopropylideneguanosine



2',3'-O-Isopropylideneguanosine (3.232g, 10mmol), cyanopentafluoro benzene (1.93g, 10mmol, 1.26ml), tetra-n-butylammoniun hydrogen sulphate (3.345g, 10 mmol) CH_2Cl_2 (50ml) and M NaOH (50ml) were stirred for ½ hr. Then an equal quantity of cyanopentafluorobenzene was added and the mixture stirred for another 1 h. The now yellow solution was treated with N HCl (60ml) with stirring and became almost colourless. The organic phase was filtered through "phase sep" paper. It was noted that there was a little solid separating during filtration - this was discarded and the organic filtrate concentrated to a foam. Treatment with EtOH afforded an initially glassy solid which gradually became more granular (and hence more tractable) after an hour of standing with occasional scraping with a spatula. The solid was recovered into a 4.25cm diam funnel by filtration and pressed to expel solvent then washed several times with EtOH and freed of solvent in vacuo over $CaCl_2$. Yield of product was 2.828g.

EXAMPLE 7

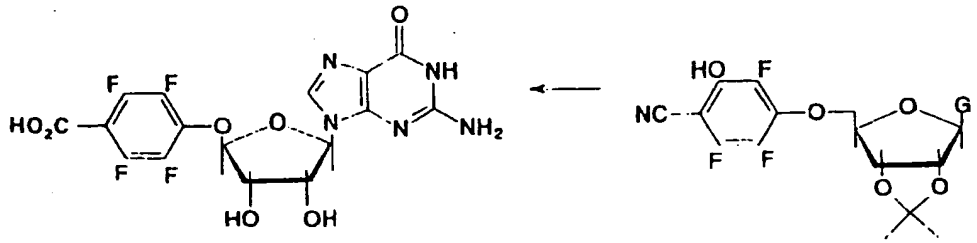
4-(2',3'-O-Isopropylideneguanosin-5'-yloxy)-2,3,5,6-tetrafluorobenzoic acid



The product from Example 6 (993mg, 2mmol) in M NaoH (20ml) was stirred at 40°C. After 7h there remained some yellow granules floating in the otherwise clear solution. These had not further dissolved in 24h, so they were removed by filtration (dry wt ca 60mg) and discarded. The solution was treated with M HCl (20 ml) whereupon a white solid separated. This was removed by filtration of the neutral solution but the filtrate came through initially as a foam which slowly aggregated to a clear liquid. When this was treated with more M HCl (5ml) further precipitate separated and this was added to the contents of the funnel and recovered (this solution was now quite acidic to pH paper). The somewhat slowly filtering mixture afforded a white solid which was washed with water and dried in vacuo over $CaCl_2$ to yield a white solid (655mg).

EXAMPLE 8

4-(Guanosin-5'-yloxy)-2,3,5,6-tetrafluorobenzoic acid

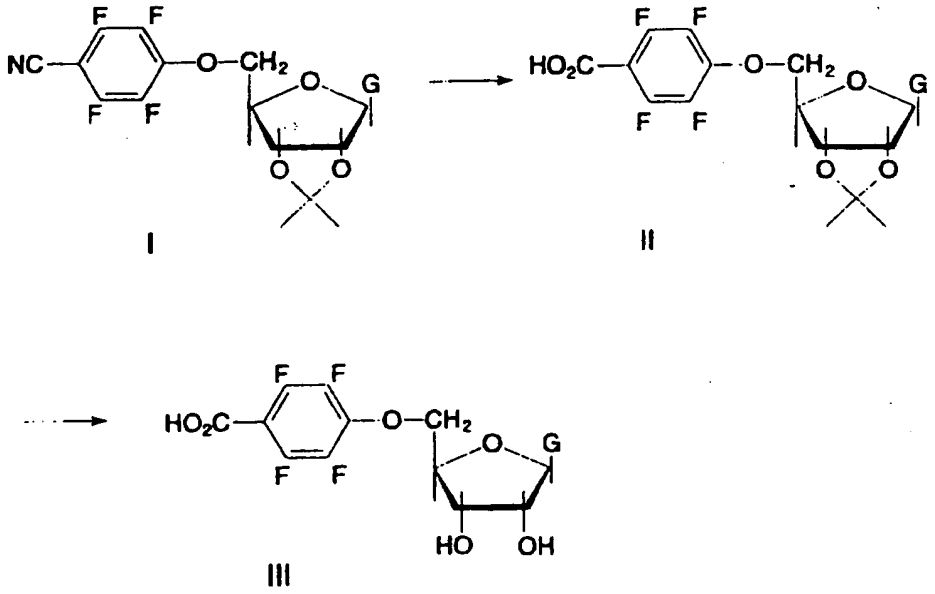


The isopropylidene derivative (see Example 7) (494mg, 1mmole) was stirred at 40°C with M aq HCl (20ml). At 1h there was substantial solid adhering to the bottom of the flask but after overnight stirring this had gone and the reaction mixture was a slightly milky solution. After a total of 24h the solution was filtered and the clear filtrate treated with M NaOH (20ml) to give a fine white precipitate which was allowed to 'age' overnight. On filtration 125mg of white solid was recovered. The neutral filtrate did not precipitate further product on further addition of alkali (5ml M NaOH). It was acidified to about pH 5 by addition of 10ml M HCl and extracted with 2x50ml EtOAC which yielded a further 70mg white solid.

EXAMPLE 9

Hydrolysis of 5'-O-(4-cyano-2,3,5,6-tetrafluoro)phenyl-2',3'-O-isopropylideneguanosine and of the product thereof: course of the reaction.

There is strong evidence that the course of these reactions are as shown in the scheme I-III and contrasts with those of Examples 1 and 2.



Two main pointers to this reaction course were:

(1) Fluorine NMR Data

For I the signals are at δ-154.1, -134.9

For II the signals are at δ-156.1, -141.4

For III the signals are at δ-155.75, -141.25

II and III were clearly grouped and distinct from I. However all form an A_2X_2 type system so the fluorines are not displaced. The larger shift could be ascribed to a pair of Fs ortho to CN in I and to CO_2H in II and III.

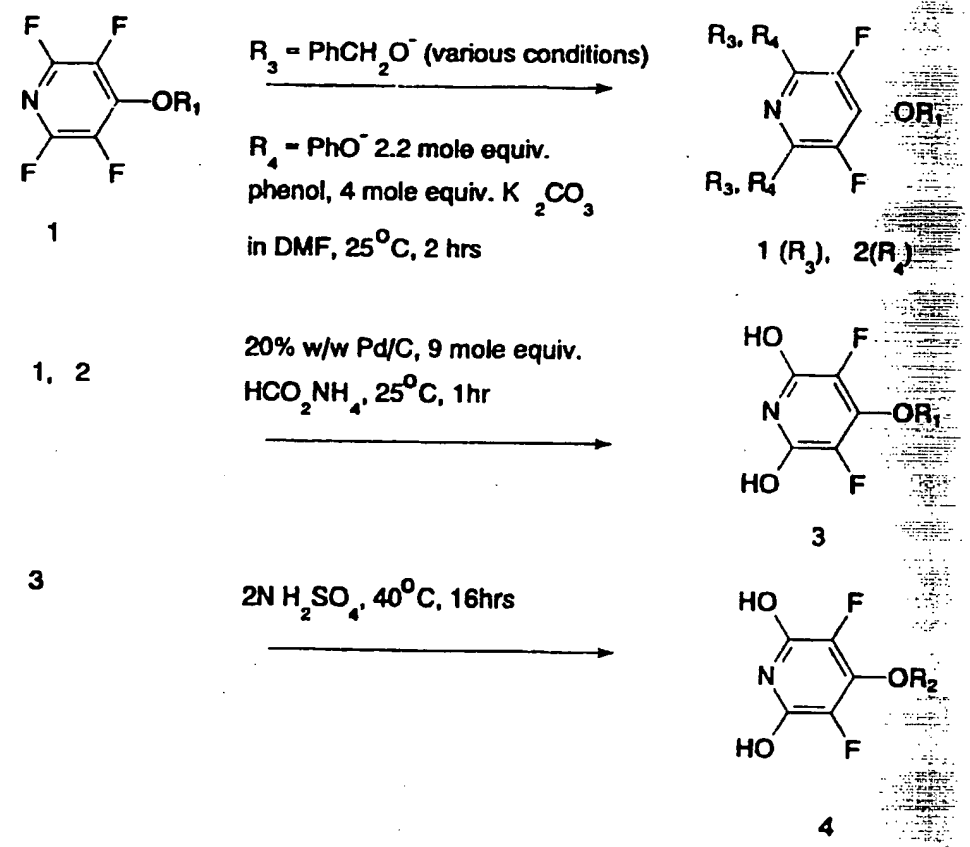
(2) IR data

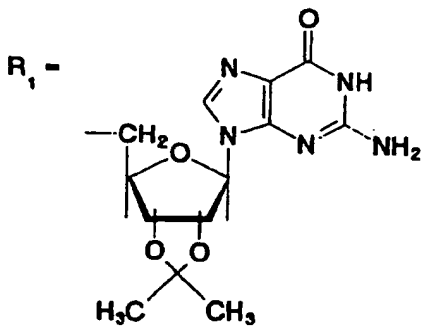
The IR spectrum of I shows a weak peak at 2154 cm^{-1} ascribable to CN stretch. This peak is absent from the spectrum of II.

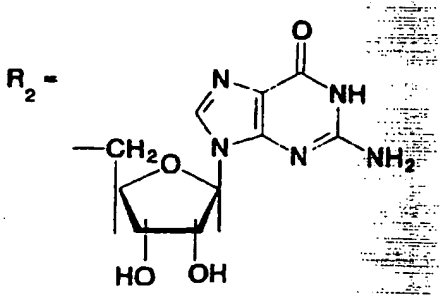
The sequence of events I→II→III→ was finally confirmed by mass spectrometry.

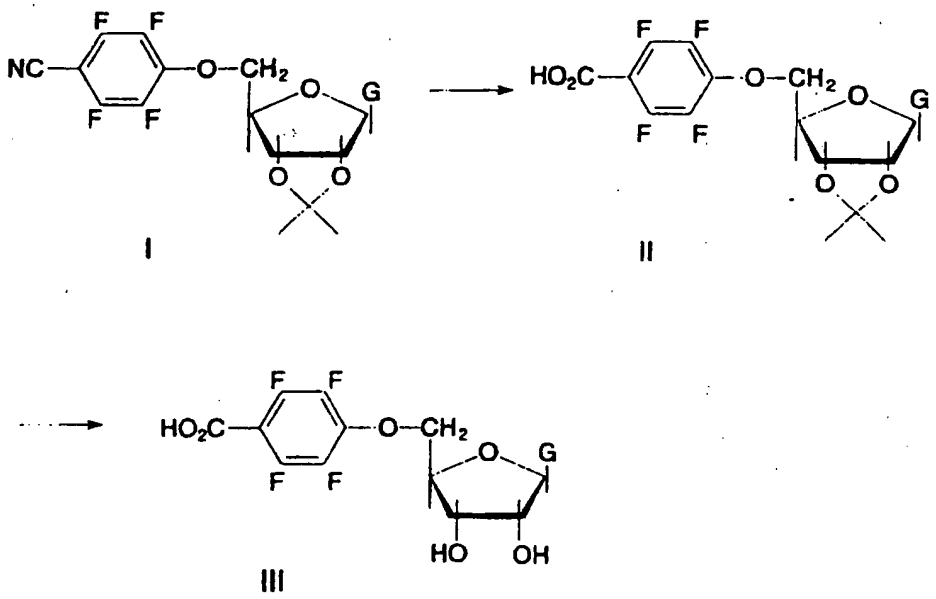
EXAMPLE 10

Compound 4 in the following scheme can be prepared by the process shown in the scheme.









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II and III were clearly grouped and distinct from I. However all form an A_2X_2 type system so the fluorines are not displaced. The larger shift could be ascribed to a pair of Fs ortho to CN in I and to CO_2H in II and III.

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The IR spectrum of I shows a weak peak at 2154 cm^{-1} ascribable to CN stretch. This peak is absent from the spectrum of II.

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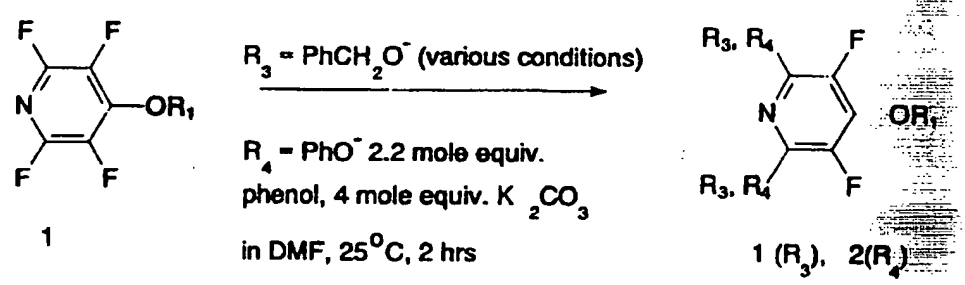
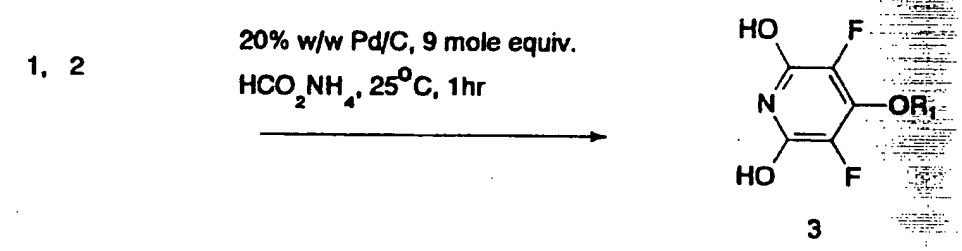
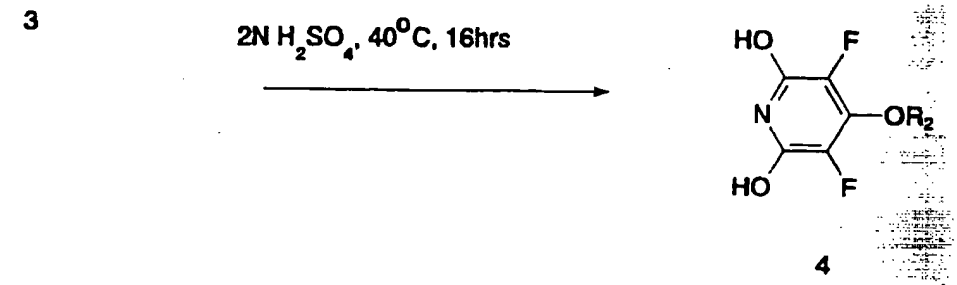
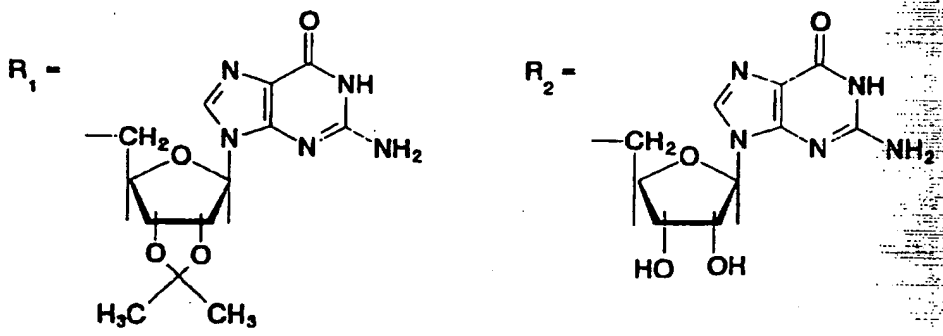
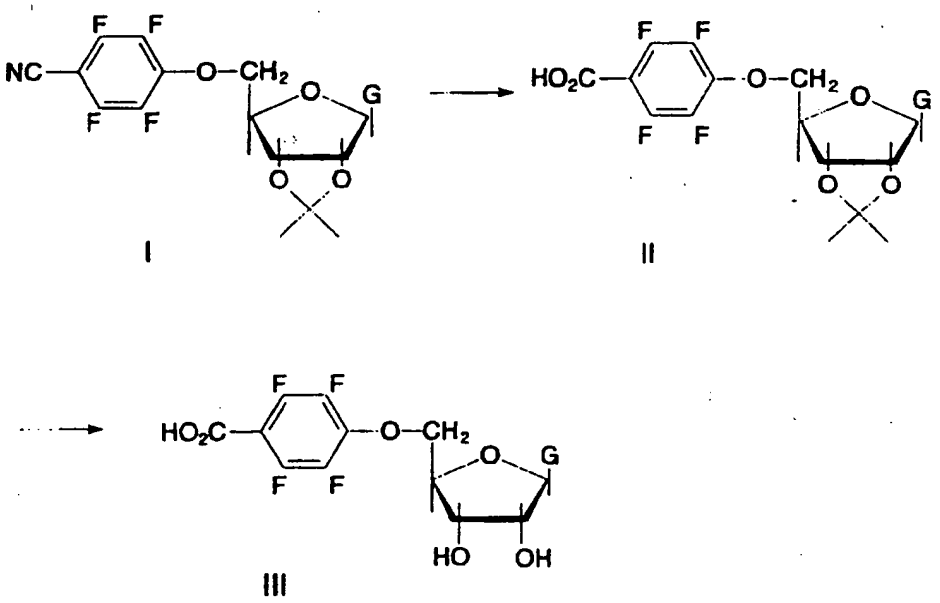










Two main pointers to this reaction course were:

(1) Fluorine NMR Data

For I the signals are at δ-154.1, -134.9

For II the signals are at δ-156.1, -141.4

For III the signals are at δ-155.75, -141.25

II and III were clearly grouped and distinct from I. However all form an A_2X_2 type system so the fluorines are not displaced. The larger shift could be ascribed to a pair of Fs ortho to CN in I and to CO_2H in II and III.

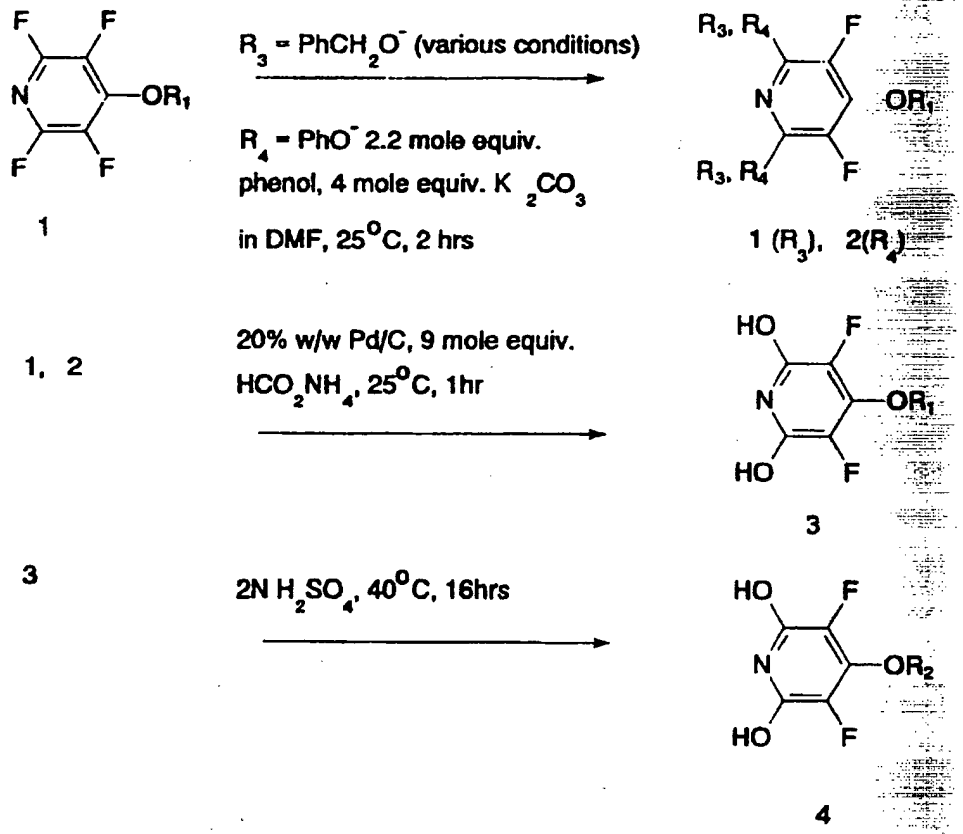
(2) IR data

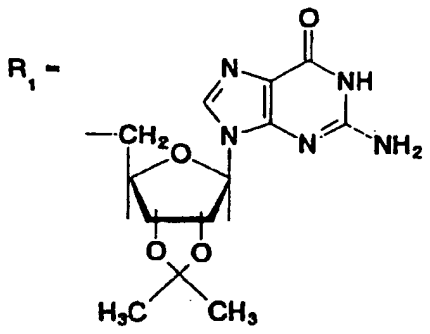
The IR spectrum of I shows a weak peak at 2154 cm^{-1} ascribable to CN stretch. This peak is absent from the spectrum of II.

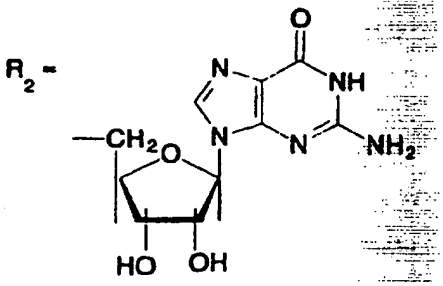
The sequence of events I→II→III→ was finally confirmed by mass spectrometry.

EXAMPLE 10

Compound 4 in the following scheme can be prepared by the process shown in the scheme.

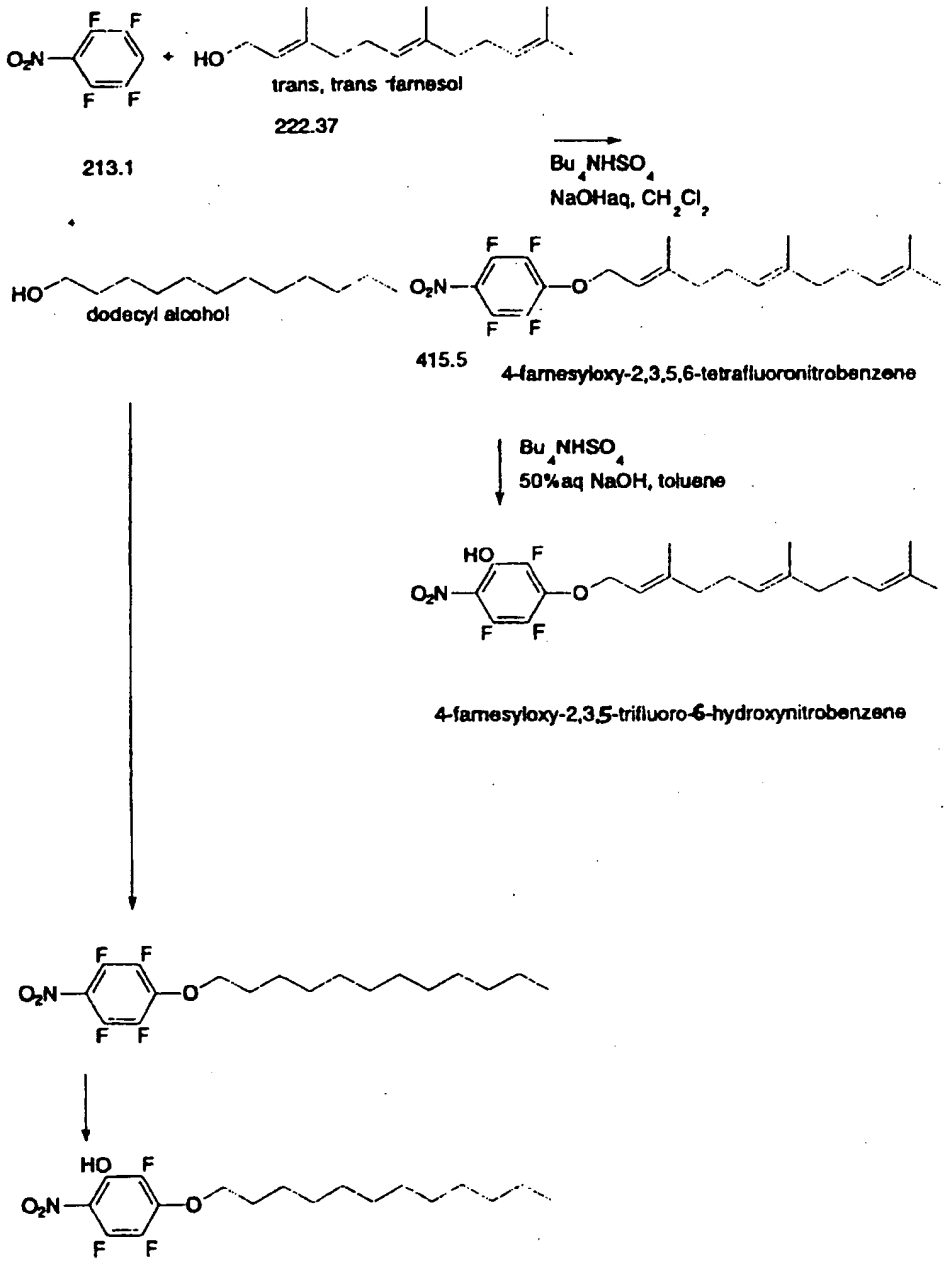






EXAMPLE 11

The following scheme illustrates the strategy used in this Example and the strategy used to make comparative compounds containing a dodecyl alcohol residue in place of the farnesyl residue.



SYNTHESIS OF 5-HYDROXY-2,3,6-TRIFLUORO-[(E,E)-3,7,11-TRIMETHYL-2,6,10-DODECATRIEN-1-OXY]-4-NITROBENZENE

To a solution of 2,3,5,6-tetrafluoro-[(E,E)-3,7,11 trimethyl-2,6,10- dodecatrien-1-oxy]-4-nitrobenzene (0.260g, 0.63 mmol) in toluene (1.5 ml) was added tetra-n-butylammonium hydrogen sulphate (0.213g, 0.63 mmol) and NaOH solution 50%w/w (0.30g 3.8 mmol). After 2 hrs stirring at 50°C the mixture was treated with conc. HCl (until pH ~ 4-5). The reaction mixture was partitioned between toluene (5 ml) and water (5 ml). This was further extracted with ether (3x10 ml), the organic phases combined, dried over $MgSO_4$ and concentrated to an oil. Flash silica chromatography, eluting with hexane-ethyl acetate 10:1 (containing a few drops of acetic acid) afforded the title compound as yellow oil (0.149g, 57%); ^{1}H-NMR (250 MHz $CDCl_3$) δ 1.59 (6H, s, CH_3), 1.68 (3H, d, J = 0.6 Hz, CH_3), 1.75 (3H, d, J = 0.6 Hz, CH_3), 2.09-1.93 (8H, m, CH_2), 4.98 (2H, d, J = 7.2 Hz, OCH_2), 5.06 (2H, m, vinylic), 5.48 (1H, t, J = 7.3 Hz, vinylic OCH_2CH); ^{19}F-NMR (235 MHz, $CDCl_3$) δ -164.50 ($1F_2$, d, J_{2-3}=22.2 Hz, meta to NO_2), -159.31 ($1F_6$, d, J_{6-2} = 7.7 Hz, meta to NO_2), -150.53 ($1F_3$, dd, J_{3-2} = 23.1 and J_{3-6} = 7.2 Hz, ortho to NO_2).

EXAMPLE 12

SYNTHESIS 5-HYDROXY-2,3,6-TRIFLUORO-[(E,E)-3,7,11- TRIMETHYL-2,6,10-DODECATRIEN-1-OXY]-4-NITROBENZENE SODIUM SALT (THE SODIUM SALT OF THE PRODUCT OF EXAMPLE 11) (CB 7721)

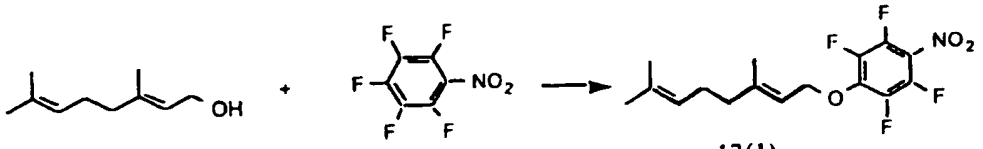
To a flask containing NaOH (0.08g, 2 mmol) was added a solution of the product of Example 11 (0.32g, 0.77 mmol) in dry ether (4 ml) and the reaction mixture was stirred for 30 min. under Argon, then filtered off and the solvent evaporated. Two crystallisations from ether followed by centrifugation to isolate the solid afforded the title compound as an orange powder (0.12g, 36%); m.p. 132-135°C; ^{1}H-NMR (250 MHz, DMSO) δ 1.54 (6H, s, CH_3), 1.62 (6H, s, CH_3), 2.08-1.91 (8H, m, CH_2), 4.58 (2H, d, J = 7.1 Hz, OCH_2), 5.06 (2H, m, vinylic), 5.39 (1H, t, J = 7.3 Hz, vinylic OCH_2CH); ^{19}F-NMR (235 MHz, DMSO) δ -182.04 ($1F_2$, dd, J_{2-3} = 28.8 and J_{2-6} = 8.8 Hz, meta to NO_2), -156.06 ($1F_6$, m, meta to NO_2), -154.8 ($1F_3$, dd, J_{3-2}= 25.2 and J_{3-6}= 8.7 Hz, ortho to NO_2); Found C

58.36, H 5.93 N 3.47, F 11.75, Na 4.79; $C_{21}H_{25}F_3N$ NaO_4 requires C 57.93, H 5.78, N 3.22, F 13.09, Na 5.28.

EXAMPLE 13

A procedure similar to Example 11 was used to make a corresponding compound in which a geranyl residue was used in place of the farnesyl residue.

1-[(E)-3,7-Dimethyl-2,6-octadien-1-yloxy]-2,3,5,6-tetrafluoro-4-nitrobenzene 13 (1)



13(1)

Tetra-n-butylammonium hydrogensulphate (0.34 g, 1 mmol) and 1 M aqueous sodium hydroxide solution (20 ml) were added to a vigorously stirred solution of geraniol (1.542 g, 10 mmol) and pentafluoronitrobenzene (2.130 g, 10 mmol) in dichloromethane (20 ml) at room temperature. After 3 h the mixture was neutralised with 1 M sulphuric acid. The dichloromethane layer was separated and the aqueous layer was extracted with dichloromethane (2 x 5 ml). The combined dichloromethane solution was washed with water (10 ml), dried ($MgSO_4$) and evaporated and the residue was chromatographed on silica (Merck no. 7729) with hexane-dichloromethane (stepwise gradient of 100:0, 40:1, 25:1, 20:1 and 15:1 in succession) as eluant. Appropiate fractions were combined and evaporated to give the compound 13 (1) as a pale yellow oil (1.354 g, 39%).

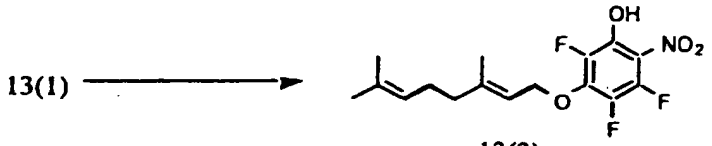
Analysis found: C, 55.14; H, 4.98; N, 3.96; F, 21.40. $C_{16}H_{17}F_4NO_3$ requires: C, 55.33; H, 4.93; N, 4.03; F, 21.88%.

NMR Spectra: δ_H($CDCl_3$ + TMS) 1.60, 1.67, 1.73 (3 x s, each 3 H, 3 x Me), 2.08 (m, 4 H, geranyl 4,5-H), 4.92 (d, J 7.3, 2 H, geranyl 1-H), 5.02 (m, 1 H, geranyl 6-H), 5.46 (t, J 7.1, 1 H, geranyl 2-H).

δ_F($CDCl_3$ + $FCCl_3$) -154.42 (m, 2 F, 2,6-F), -147.82 (m, 2 F, 3,5-F).

Mass Spectrum: m/z (FAB$^+$) 346 [$(M-1)^+$, 21%], 137 [$C_{10}H_{17}^+$, 100%].

1-[(E)-3,7-Dimethyl-2,6-octadien-1-yloxy]-2,3,6-trifluoro-5-hydroxy-4-nitrobenzene 13 (2) (= CB 7741)



13(2)

A mixture of 1-[(E)-3,7-dimethyl-2,6-octadien-1-yloxy]-2,3,5,6-tetrafluoro-4-nitrobenzene 13 (1), (0.989 g, 2.85 mmol), toluene (7.5 ml), tetra-n-butylammonium hydrogensulphate (0.97 g, 2.9 mmol), and 50% aqueous sodium hydroxide solution (1.36 g, 17 mmol) was rapidly stirred at 50 °C under argon for 1 h. It was then cooled and partitioned between toluene (30 ml) and water (20 ml) with acidification (2 M hydrochloric acid) of the aqueous phase to pH 1.5 - 2. The aqueous layer was extracted with ether (3 x 15 ml) and the combined organic solution was washed with water (4 x 10 ml), dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica (Merck no. 7729), eluting successively with hexane-dichloromethane (stepwise gradient: 100:0, 4:1, 2:1 and 1:1) and dichloromethane-ethanol (stepwise gradient: 100:0, 99:1, 98:2, and 95:5). Appropriate fractions were combined and evaporated to give the title compound 13 (2) as a yellow oil (0.609 g, 62%).

Analysis found: C, 55.99; H, 5.45; N, 3.90; F, 16.24

$C_{16}H_{18}F_3NO_4$ requires: C, 55.65; H, 5.25; N, 4.06; F, 16.51%.

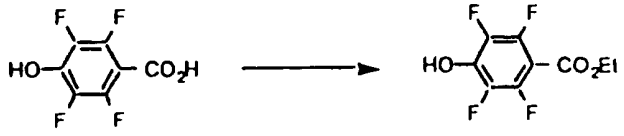
NMR Spectra: δ_H($CDCl_3$ + TMS) 1.59, 1.66, 1.74 (3 x s, each 3 H, 3 x Me), 2.08 (m, 4 H, geranyl 4,5-H), 4.98 (d, J 7.3, 2 H, geranyl 1-H), 5.03 (m, 1 H, geranyl 6-H), 5.47 (t, J 7.1, 1 H, geranyl 2-H), 10.50 (br. s, 1 H, OH).

δ_F($CDCl_3$ + $FCCl_3$) -162.17 (d, J 23, 1 F, 2-F), -155.49 (d, J 9, 1 F, 6-F), -146.80 (dd, J 8, 22, 1 F, 3-F).

EXAMPLE 14

This example illustrates the preparation of a compound in which X and Y are each F and Z is -C(R) where R is carboxy.

Ethyl 2,3,5,6-tetrafluoro-4-hydroxybenzoate 14 (1)



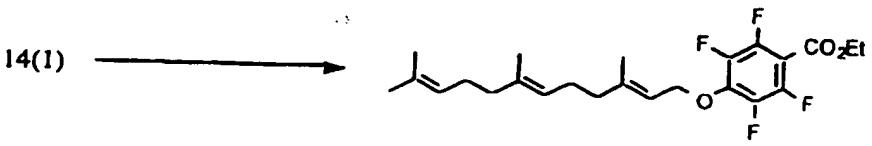
14(1)

A mixture of 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid hydrate (2 g, 8.8 mmol), ethanol (10 ml), and concentrated sulphuric acid (4 ml) was stirred and heated under reflux under argon for 17.5 h. The products were cooled and poured into a stirred, ice-cold mixture of water (100 ml) and diethyl ether (100 ml). The aqueous layer was extracted with ether (5 x 50 ml) and the combined ethereal solution was washed with saturated aqueous sodium hydrogen carbonate (100 ml). The $NaHCO_3$ solution was acidified to pH 4 with 1 M sulphuric acid and back-extracted with ether (4 x 50 ml). All the ethereal solutions were combined, washed with water (50 ml), dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica (Merck no. 15111) using dichloromethane-ethanol (stepwise gradient: 100 : 0, 99 : 1, 250 : 6, 250 : 8, 250 : 13, and 9 : 1) as eluant to give the title compound 14 (1) as a white solid (1.473 g, 71%), mp 108-110 °C [lit. (J. Org. Chem. USSR, 1989, 25(2), 317) 107-109 °C].

NMR Spectra: δ_H($CDCl_3$ + TMS) 1.39 (t, J 7.2, 3 H, CH_3), 4.42 (q, J 7.1, 2 H, CH_2), 6.3 (br. s, 1 H, OH).
δ_F($CDCl_3$ + $FCCl_3$) -163.12 (m, 2 F, 3,5-F), -140.54 (m, 2 F, 2,6-F).

Mass Spectrum: m/z (FAB^+) 239 [$(M+H)^+$, 100%].

Ethyl 2,3,5,6-tetrafluoro-4-(*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy)benzoate 14 (2)



14(2)

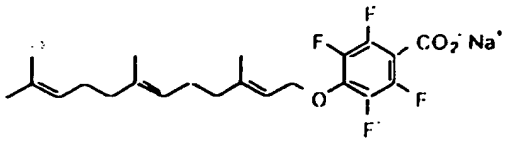
Caesium carbonate (0.82 g, 2.5 mmol) was added to a stirred solution of ethyl 2,3,5,6-tetrafluoro-4-hydroxybenzoate (1) (0.50 g, 2.1 mmol) and *trans, trans*-farnesyl bromide (0.66 g, 2.3 mmol) in dry N,N-dimethylformamide (5 ml) under argon at room temperature. The mixture was protected from light. After 16 h the mixture was evaporated and the residue partitioned between ethyl acetate (25 ml) and half-saturated brine (25 ml). The aqueous layer was extracted with ethyl acetate (3 x 10 ml) and the combined ethyl acetate solution washed with water (10 ml), dried ($MgSO_4$) and evaporated. The residue was redissolved in ether (50 ml), washed with half-saturated brine (3 x 10 ml), dried ($MgSO_4$) and again evaporated, then chromatographed on silica (Merck no. 15111) using hexane-dichloromethane (stepwise gradient: 9 : 1, 3 : 1, 2 : 1) as eluant to give the title compound 14 (2) as a colourless oil (0.794 g, 85%).

Analysis found: C, 65.35; H, 6.89; N, 0.00; F, 16.92.
$C_{24}H_{30}F_4O_3$ requires: C, 65.15; H, 6.83; N, 0.00; F, 17.17%.

NMR Spectra: $\delta_H(CDCl_3)$ 1.39 (t, J 7.1, 3 H, $\underline{CH_3}CH_2$), 1.59 (s, 6 H), 1.68 (s, 3 H), 1.71 (s, 3 H) (4 x Me), 2.07 (m, 8 H, 4,5,8,9-CH_2), 4.42 (q, J 7.0, 2 H, $CH_3\underline{CH_2}$), 4.84 (d, J 7.2, 2 H, 1-CH_2), 5.07 (m, 2 H, 6,10-CH), 5.47 (t, J 7.1, 1 H, 2-CH).
$\delta_F(CDCl_3 + FCCl_3)$ -156.26 (m, 2 F, 3,5-F), -141.05 (m, 2 F, 2,6-F).

Mass Spectrum: m/z (FAB^+) 442 (M^+).

2,3,5,6-Tetrafluoro-4-(*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy)benzoic acid, sodium salt 14 (3) (=CB 7745)

14(2) ⟶ 

14(3)

20% Aqueous sodium hydroxide solution (0.1 ml) was added to a rapidly stirred solution of ethyl 2,3,5,6-tetrafluoro-4-(*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy)benzoate 14 (2) (0.115 g, 0.26 mmol) in ethanol (1.1 ml) and water (0.1 ml) at room temperature with protection from light. After 2.5 h the mixture was diluted with water (6 ml), cooled in ice and centrifuged. The supernatant was decanted off and the precipitated solid was washed with water (2 x 6 ml) by resuspension, centrifugation and decantation, then crystallised from water (5 ml) to give the title compound 14 (3) (0.062 g, 55%) as colourless plates, mp 224 226 °C.

Analysis found: C, 59.80; H, 5.87; N, 0.04.
$C_{22}H_{25}F_4NaO_3$. $0.25H_2O$ requires C, 59.93; H, 5.83; N, 0.00%.

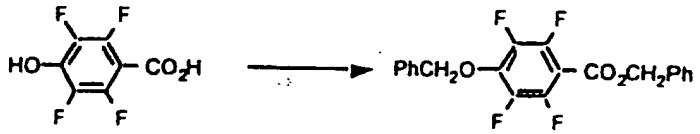
NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.56 (s, 6 H, 11-Me), 1.62, 1.63 (2 x s, each 3 H, 3,7-Me), 1.96, 2.02 (2 x m, total 8 H, 4,5,8,9-CH_2), 4.64 (d, J 7.3, 2 H, 1-CH_2), 5.07 (m, 2 H, 6,10-CH), 5.42 (t, J 7.0, 1 H, 2-CH).
$\delta_F(CD_3SOCD_3 + FCCl_3)$ -156.76 (m, 2 F, 3,5-F), -145.37 (m, 2 F, 2,6-F).

Mass Spectrum: m/z (ESI, -ve ion mode) 413.2 [$(M-Na)^-$, 14%], 369.2 [$(M-Na-CO_2)^-$, 100%].

EXAMPLE 15

Using the same starting materials as in Example 14 analogous compounds may be made in which one of X and Y is hydroxy and the other is fluoro.

Benzyl 4-benzyloxy-2,3,5,6-tetrafluorobenzoate 15 (3)



15(3)

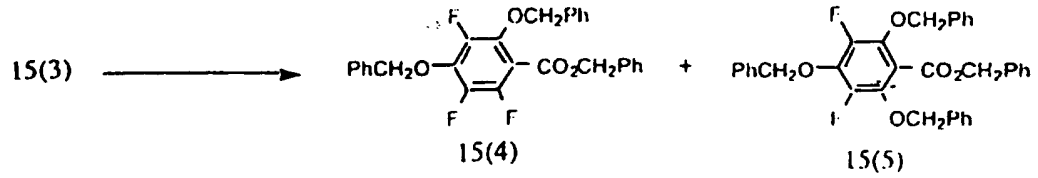
Caesium carbonate (30.4 g, 93 mmol) was added to a stirred mixture of 2,3,5,6-tetrafluoro-4-hydroxybenzoic acid hydrate (Aldrich Chemical Co.) (9.48 g, 41.6 mmol), dry N,N-dimethylformamide (80 ml) and benzyl bromide (11.9 ml, 100 mmol) under argon at room temperature. After 28 h the mixture was evaporated to dryness and the residue partitioned between ethyl acetate (150 ml) and water (50 ml). The aqueous layer was extracted with ethyl acetate (3 x 50 ml) and the combined ethyl acetate solution washed with half-saturated brine (2 x 30 ml), dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica (Merck no. 15111) using hexane-dichloromethane (stepwise gradient: 100:0, 9:1, 4:1, 3:1 and 12:5 in succession). Evaporation of appropriate fractions left the title compound 15 (3) as a white solid (16.3 g, quantitative yield), mp (after recrystallisation from hexane) 65-67 ^{o}C.

Analysis found: C, 64.58; H, 3.72; N, 0.00; F, 19.50.
$C_{21}H_{14}F_4O_3$ requires: C, 64.62; H, 3.62; N, 0.00; F, 19.47%.

NMR Spectra: δ_H($CDCl_3$ + TMS) 5.34, 5.38 (2 x s, each 2 H, 2 x CH_2), 7.39 (m, 10 H, 2 x Ph).
δ_F($CDCl_3$ + $FCCl_3$) -155.73 (m, 2 F, 3,5-F), -140.08 (m, 2 F, 2,6-F).

Mass Spectrum: m/z (FAB$^+$) 413 [(M+Na)$^+$, 63 %], 389 [(M-1), 100%].

Benzyl 2,4-di-(benzyloxy)-3,5,6-trifluorobenzoate 15 (4) and benzyl 2,4,6-tri-(benzyloxy)-3,5-difluorobenzoate 15 (5).



15(4) 15(5)

Dry benzyl alcohol (1.7 ml, 16 mmol) was added to a stirred suspension of potassium tert-butoxide (1.15 g, 10 mmol) in dry THF (25 ml) under argon at room temperature. After 20 min the mixture was cooled to 10 °C and after a further 10 min, a solution of benzyl 4-benzyloxy-2,3,5,6-tetrafluorobenzoate 15 (3) (3.148 g, 8 mmol) in THF (10 ml) was added rapidly by syringe and the mixture was allowed to come to room temperature. After a further 45 min acetic acid (1 ml) was added. The mixture was filtered and the solid material washed with dichloromethane. The combined filtrate and washings were evaporated and the residue was partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (25 ml). The aqueous layer was extracted with dichloromethane (4 x 10 ml) and the combined dichloromethane solution was washed with half-saturated brine (100 ml), dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica (Merck no. 7729) using hexane-dichloromethane (100:0, 2:1 and 1:1 in succession) as eluants. Appropriate fractions were combined and evaporated to give (in order of elution) compound 15 (4) (2.257 g, 58%) and compound 15 (5) (0.674 g, 15%) as colourless oils.

Data for compound 15 (4):

Analysis found: C, 70.60; H, 4.60; N, 0.01; F, 11.60.
$C_{28}H_{21}F_3O_4$ requires: C, 70.29; H, 4.42; N, 0.00; F, 11.91%.

NMR Spectra: δ_H($CDCl_3$ + TMS) 5.02, 5.24, 5.29 (3 x s, each 2 H, 3 x CH_2), 7.31 (m, 15 H, 3 x Ph).

δ_F($CDCl_3$ + $FCCl_3$) -155.86 (d, J 23, 1 F, 5-F), -147.46 (d, J 10, 1 F, 3-F), -141.97 (dd, J 10, 24, 1 F, 6-F).

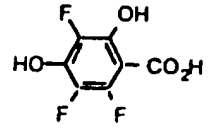
Mass Spectrum: m/z (FAB^+) 501 [$(M+Na)^+$, 100%], 479 [$(M+H)^+$, 44%].

Data for compound 15 (5):

Analysis found: C, 74.17; H, 5.08; N, 0.00; F, 6.75.
$C_{35}H_{28}F_2O_5$ requires: C, 74.19; H, 4.98; N, 0.00; F, 6.71%.

NMR Spectra: δ_H($CDCl_3$ + TMS) 5.00 (s, 4 H, 2 x CH_2), 5.18, 5.21 (2 x s, each 2 H, 2 x CH_2), 7.31 (m, 20 H, 4 x Ph).
δ_F($CDCl_3$ + $FCCl_3$) -147.93 (s).

2,3,5-Trifluoro-4,6-dihydroxybenzoic acid 15 (6)

15(4) ⟶ 

15(6)

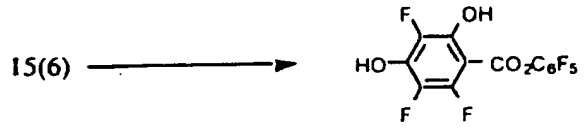
A mixture of benzyl 2,4-di-(benzyloxy)-3,5,6-trifluorobenzoate 15 (4) (2.2 g, 4.6 mmol), methanol (366 ml), water (0.7 ml), and 10% Pd-C (1.1 g) was stirred under hydrogen (balloon) at room temperature for 3 h. The catalyst was removed by filtration and the solution evaporated. The residue was triturated with hexane, dried and crystallised from water to give the title compound 15 (6) as colourless plates (0.778 g, 81%), mp 215-220 °C (with sublimation).

Analysis found: C, 40.27; H, 1.50; N, 0.03; F, 27.50.
$C_7H_3F_3O_4$ requires: C, 40.40; H, 1.45; N, 0.00; F, 27.39%.

NMR Spectra: d_H(CD_3SOCD_3) 11.76 (br. s).
d_F(CD_3SOCD_3 + $FCCl_3$) -168.96 (d, J 23.6, 1 F, 3-F), -162.04 (d, J 10.0, 1 F, 5-F), -139.75 (dd, J 9.9, 23.6, 1 F, 2-F).

Mass Spectrum: m/z (FAB^+) 209 [$(M+H)^+$, 5%], 191 (53%), 154 (100%).

Pentafluorophenyl 2,3,5-trifluoro-4,6-dihydroxybenzoate 15 (7)



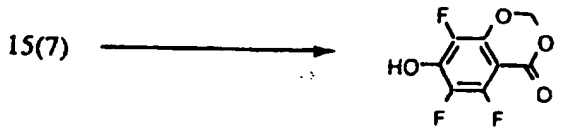
15(7)

Pentafluorophenyl trifluoroacetate (0.92 ml, 5.4 mmol) was added to a stirred mixture of 2,3,5-trifluoro-4,6-dihydroxybenzoic acid 15 (6) (1.073 g, 5.2 mmol), dry dichloromethane (39 ml), and pyridine (1.3 ml, 16 mmol) under argon at room temperature. After 4 h further pentafluorophenyl trifluoroacetate (0.7 ml, 4 mmol) was added and after a further 12 h the mixture was diluted with dichloromethane (100 ml), washed successively with 0.1 M hydrochloric acid (3 x 30 ml) and water (2 x 30 ml), dried ($MgSO_4$) and evaporated. The residue was dried in vacuo, triturated with hexane and further dried to give the title compound 15 (7) (1.813 g, 94%) as a white solid, mp 125-130 ^oC, which was used without further purification.

NMR Spectrum: δ_F(CD_3SOCD_3 + $FCCl_3$) -167.41 (d, J 23.6, 1 F, 3-F), -162.08 (t, J 22.0, 2 F, pentafluorophenyl 3,5-F), -157.99 (d, J 7.8, 1 F, 5-F), -157.31 (t, J 22.9, 1 F, pentafluorophenyl 4-F), -152.70 (d, J 20.9, 2 F, pentafluorophenyl 2,6-F), -141.05 (dd, J 8.2, 23.0, 1 F, 2-F).

Mass Spectrum: m/z (FAB^+) 375 [$(M+H)^+$, 5%], 191 (100%). Measured molecular mass = 374.9920; actual molecular mass [$(C_{13}H_3F_8O_4)^+$] = 374.9904.

5,6,8-Trifluoro-7-hydroxy-4H-1,3-benzodioxin-4-one 15 (8)



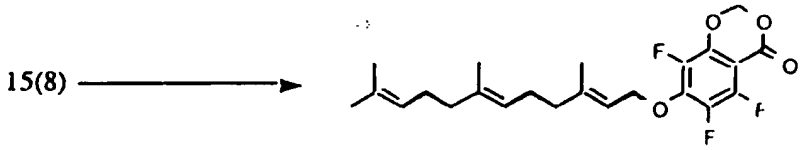
15(7) 15(8)

A suspension of paraformaldehyde (0.48 g) in dry THF (11 ml) was stirred under argon at 25 °C for 5 min. Pentafluorophenyl 2,3,5-trifluoro-4,6-dihydroxybenzoate 15 (7) (1.2 g, 3.2 mmol) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.72 g, 6.4 mmol) were then added successively. After 21 h sufficient ethanol was added to dissolve most of the undissolved material present and the mixture was preadsorbed onto silica (Merck no. 7729) (4 g) and chromatographed with dichloromethane-ethanol (stepwise gradient: 100:0, 97:3, 95:5) as eluant. Evaporation of appropriate fractions and trituration of the residue with hexane left a white solid (0.268 g) containing the title compound 15 (8) and DABCO (detected by NMR and TLC). This material (0.257 g) was dissolved in methanol-water (1:1 v/v; 12 ml) and passed through Biorad AG 50W-X4 100-200 mesh cation exchange resin (H^+ form; bed volume 11 ml), using the same solvent mixture as eluant. The eluate was evaporated to low volume resulting in crystallisation of the title compound 15 (8) which was collected after cooling and dried; yield 0.203 g, 30%; mp 214 °C.

Analysis found: C, 43.65; H, 1.36; N, 0.00; F, 26.09.
$C_8H_3F_3O_4$ requires: C, 43.66; H, 1.37; N, 0.00; F, 25.89%.
NMR Spectra: $\delta_H(CD_3SOCD_3-D_2O)$ 5.82 (s, CH_2).
$\delta_F(CD_3SOCD_3-D_2O + FCCl_3)$ -163.09 (d, J 25.6, 1 F, 6-F), -159.70 (d, J 6.8, 1 F, 8-F), -140.22 (dd, J 10.7, 21.1, 1 F, 5-F).

Mass Spectrum: m/z (FAB^+) 221 [$(M+H)^+$, 100%], 191 (65%).

5,6,8-Trifluoro-7-(*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy)-4H-1,3-benzodioxin-4-one 15 (9)



15(9)

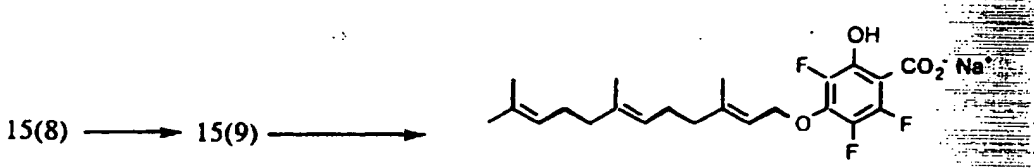
trans,trans-Farnesyl bromide (0.15 g, 0.53 mmol) was added to a stirred mixture of 5,6,8-trifluoro-7-hydroxy-4H-1,3-benzodioxin-4-one 15 (8) (0.075 g, 0.34 mmol), caesium carbonate (0.167 g, 0.51 mmol), and dry N,N-dimethylformamide (0.75 ml) under argon at room temperature. The mixture was protected from light. After 2 h the mixture was evaporated and toluene (15 ml) was added and evaporated. The residue was chromatographed on silica (Merck no. 7729) using hexane-ethyl acetate (stepwise gradient: 100 : 0, 99.5 : 0.5, 99 : 1, 97 : 3, 95 : 5) as eluant to give the title compound 15 (9) (0.083 g, 57%) as a colourless oil which solidified when cooled.

Analysis found: C, 64.79; H, 6.46; N, 0.02; F, 13.33.
$C_{23}H_{27}F_3O_4$ requires: C, 65.08; H, 6.41; N, 0.00; F, 13.43%.

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.55 (s, 6 H, 11-Me), 1.63, 1.68 (2 x s, each 3 H, 3,7-Me), 2.04 (m, 8 H, farnesyl 4,5,8,9-CH_2), 4.91 (d, J 7.1, 2 H, farnesyl 1-CH_2), 5.04 (m, 2 H, farnesyl 6,10-CH), 5.45 (t, J 7.1, 1 H, farnesyl 2-CH), 5.88 (s, 2 H, 2-CH_2).
$\delta_F(CD_3SOCD_3 + FCCl_3)$ -158.73 (d, J 22.9, 1 F, 6-F), -154.51 (d, J 11.2, 1 F, 8-F), -139.63 (dd, J 11.9, 22.0, 1 F, 5-F).

Mass Spectrum: m/z (FAB$^+$) 447 [(M+Na)$^+$, 96%], 423 [(M-1)$^+$, 100%].

2,3,5-Trifluoro-6-hydroxy-4-(*trans,trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy)benzoic acid, sodium salt 15 (10) (=CB7744)



15(10)

trans,trans-Farnesyl bromide (0.16 g, 0.56 mmol) was added to a stirred mixture of 5,6,8-trifluoro-7-hydroxy-4H-1,3-benzodioxin-4-one 15 (8) (0.100 g, 0.45 mmol), caesium carbonate (0.183 g, 0.56 mmol) and dry N,N-dimethylformamide (0.95 ml) under argon at 25 °C. The mixture was protected from light. After 1 h, TLC [$CHCl_3$-MeOH-AcOH (95:5:1)] indicated that most of the starting material had been converted to compound 15 (9). The mixture was stirred in the dark for a further 21 h after which the initial product 15 (9) had been converted to a more polar product. The mixture was evaporated and the residue chromatographed on silica (Merck no. 7729) using dichloromethane-ethanol (stepwise gradient: 100:0, 98:2, 97:3, 95:5, 90:10, and 85:15) as eluant. The product fractions [analysed by TLC with $CHCl_3$-MeOH-AcOH (95:5:3) as eluant and iodine vapour as visualisation reagent] that were free of a low-R_F impurity were combined and evaporated to leave a glass (0.079 g). This material was dissolved in ethanol-water (4:1 v/v) and passed through a 10 cm x 0.5 cm column of Amberlite IRC 50 (Na^+) cation exchange resin using further ethanol-water (4:1 v/v) as eluant. The eluate was evaporated to dryness and the residue triturated with cold diethyl ether to give a white solid which was isolated by decantation and dried (yield 0.064 g). This material (0.042 g) was crystallised from water to give the title compound 15 (10) (0.022 g, 17% based on 15 (8), mp 202-203 °C.

Analysis found: C, 60.55; H, 6.04; N, 0.00; F, 13.21.

$C_{22}H_{26}F_3NaO_4$ requires: C, 60.82; H, 6.03; N, 0.00; F, 13.12%.

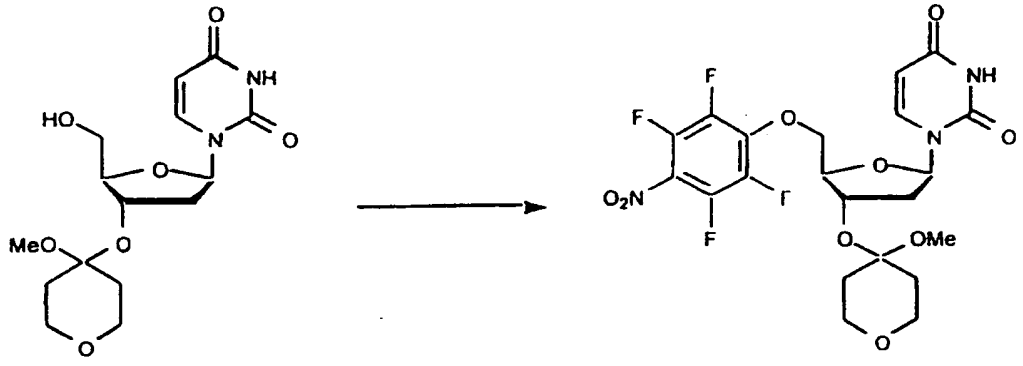
NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.55 (s, 6 H, 11-Me), 1.63 (s, 6 H, 3,7-Me), 1.95, 2.01 (2 x m, total 8 H, 4,5,8,9-CH_2), 4.64 (d, J 7.2, 2 H, 1-CH_2), 5.06 (m, 2 H, 6,10-CH), 5.41 (t, J 7.3, 1 H, 2-CH), 11.47 (s, 1 H, OH).
$\delta_F(CD_3SOCD_3 + FCCl_3)$ -172.14 (d, J 22.4, 1 F, 3-F), -159.99 (t, J 6.8, 1 F, 5-F), -144.90 (dd, J 13.1, 24.0, 1 F, 2-F).

Mass Spectrum: m/z (ESI, -ve ion mode) 411.1 [$(M-Na)^-$, 100%], 367.2 [$(M-Na-CO_2)^-$, 68%].

EXAMPLE 16

Further compounds were made in which the biologically active compound to which the phosphate mimicking moiety was attached was a compound based on deoxyuridine.

3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine 16(2)



16(1) 16(2)

3'-O-(4-Methoxytetrahydropyran-4-yl)-2'-deoxyuridine 16 (1) (Y. Ji, W. Bannwarth and B. Luu, Tetrahedron, 1990, 46, 487) (0.68 g, 2 mmol), pentafluoronitrobenzene (0.51 g, 2 mmol), tetra-n-butylammonium hydrogensulphate (0.68 g, 2 mmol), dichloromethane (10 ml) and 1 M aqueous sodium hydroxide solution (10 ml) were stirred rapidly together at room temperature. After 35 min the mixture was diluted with dichloromethane (40 ml) and the dichloromethane layer was

separated. The aqueous layer was diluted with water (40 ml) and extracted with dichloromethane (5 x 10 ml). The combined dichloromethane solution was washed with water (3 x 10 ml), dried ($MgSO_4$) and evaporated onto silica (Merck no. 7729; 5 g). Chromatography with dichloromethane-ethanol (stepwise gradient: 100:0, 250:5, 250:7, 250:13) and evaporation of appropriate fractions afforded a glass (0.644 g). This material (0.628 g) was crystallised from acetonitrile to give the title compound 16 (2) (0.336 g, 32%) mp 162-164 °C.

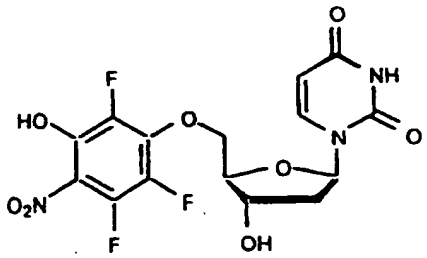
Analysis found: C, 47.20; H, 3.98; N, 7.91; F, 14.24. $C_{21}H_{21}F_4N_3O_9$ requires: C, 47.11; H, 3.95; N, 7.85; F, 14.19%.

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.78 (m, 4 H, 3'',5''-H), 2.32 (m, 2 H, 2'-H), 3.15 (s, 3 H, Me), 3.47, 3.62 (2 x m, each 2 H, 2'',6''-H), 4.23 (m, 1 H, 4'-H), 4.58 (m, 1 H, 3'-H), 4.67 (m, 2 H, 5'-H), 5.63 (d, J 8.0, 1 H, 5-H), 6.18 (t, J 6.8, 1 H, 1'-H), 7.64 (d, J 8.2, 1 H, 6-H), 11.39 (s, 1 H, N3-H). $\delta_F(CD_3SOCD_3 + FCCl_3)$ -155.11 (m, 2 F, 2,6-F), -146.87 (m, 2 F, 3,5-F).

Mass Spectrum: m/z (FAB^+) 536 (M^+, 14%), 404 (100%).

5'-O-(2,3,6-Trifluoro-5-hydroxy-4-nitrophenyl)-2'-deoxyuridine 16 (4) (=CB7746)

16(2) ⟶



16(4)

3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine 16 (2) (0.050 g, 0.09 mmol) was stirred with 0.1 M aqueous sodium hydroxide solution (4.7 ml) at room temperature. After 40 h the

solution was adjusted to pH 8.5 with acetic acid, then concentrated completely. A solution of the residue in acetic acid (4 ml) and water (1 ml) was stirred at room temperature for 22 h, kept at 5 °C for a further 44 h, and evaporated. The residue was triturated with diethyl ether and dried. A solution of the resulting orange solid in methanol-water (1 : 1 v/v) was passed through a column (120 x 11 mm; bed volume 11 ml) of Biorad AG 50W-X4 100-200 mesh cation exchange resin (H^+ form) using further methanol-water (1 : 1 v/v) as eluant. The eluate was partially evaporated resulting in crystallisation of the title compound 16 (4) which was collected after cooling, washed with water and dried (yield 0.029 g, 74%; mp 174-176 °C).

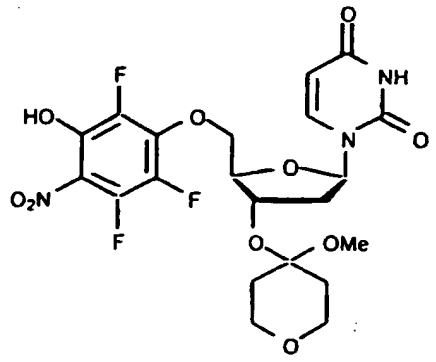
Analysis found: C, 41.56; H, 3.04; N, 9.58.
$C_{15}H_{12}F_3N_3O_8$. $0.75H_2O$ requires: C, 41.63; H, 3.14; N, 9.71%.

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 2.20 (m, 2 H, 2'-H), 4.05 (m, 1 H, 4'-H), 4.34 (m, 1 H, 3'-H), 4.48 (m, 2 H, 5'-H), 5.4 (br. s, 1 H, 3'-OH), 5.60 (dd, J 2.1, 8.1, 1 H, 5-H), 6.20 (t, J 6.8, 1 H, 1'-H), 7.62 (d, J 8.0, 1 H, 6-H), 11.29 (s, 1 H, N3-H). $\delta_F(CD_3SOCD_3-D_2O + FCCl_3)$ -163.19 (br. m, 1 F, 2-F), -151.92 (d, J 6.2, 1 F, 6-F), -151.70 (dd, J 6.7, 23.7, 1 F, 3-F).

Mass Spectrum: m/z (FAB^+) 442 [$(M + Na)^+$, 36%], 420 [$(M + H)^+$, 57%].
Measured molecular mass = 420.0640; actual molecular mass [$(M + H)^+$] = 420.0655.

3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)-2'-deoxyuridine 16 (3)

16(2) ⟶



16(3)

This compound is the product of the first step of the above two-step procedure, where it is not isolated; it may be isolated as follows: 3'-O-(4-methoxytetrahydropyran-4-yl)-5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine 16 (2) (0.100 g, 0.18 mmol) was stirred with 0.1 M aqueous sodium hydroxide solution (9.4 ml) at room temperature. After 44 h the solution was adjusted to pH 5 with acetic acid then concentrated until a solid separated. After cooling at 5 °C overnight the solid was collected, washed with water and dried to give the title compound 16 (3) (0.092 g, 92%), mp 158-160 °C.

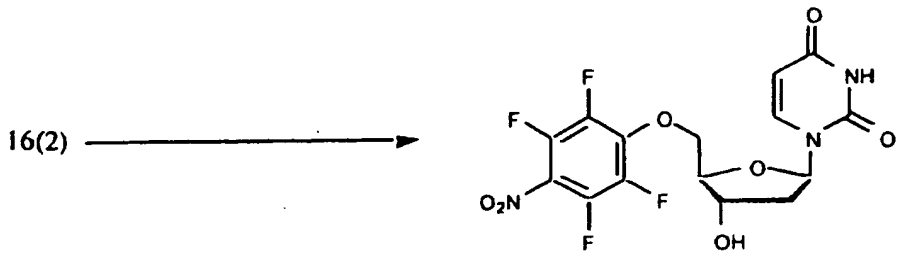
Analysis found: C,47.08; H,4.09; N,7.77.
$C_{21}H_{22}F_3N_3O_{10}$ requires: C, 47.29; H, 4.16; N, 7.88.

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.75 (m, 4 H, 3'', 5''-H), 2.32 (t, J 6.3, 2 H, 2'-H), 3.15 (s, 3 H, Me), 3.49, 3.62 (2 x m, each 2 H, 2'',6''-H), 4.22 (m, 1 H, 4'-H), 4.51 (m, 2 H, 5'-H), 4.57 (m, 1 H, 3'-H), 5.62 (dd, J 2.0, 8.1, 1 H, 5-H), 6.19 (t, J 6.8, 1 H, 1'-H), 7.63 (d, J 8.0, 1 H, 6-H), 11.31 (s, 1 H, N3-H).
$\delta_F(CD_3SOCD_3 + FCCl_3)$ -163.52 (m, 1 F), -151.94 (s, 1 F), -151.85 (s, 1 F).

Mass Spectrum: m/z (FAB^+) 556 [$(M + Na)^+$, 25%], 534 [$(M + H)^+$, 24%].

Measured molecular mass = 534.1350; actual molecular mass [(M + H)$^+$] = 534.1336.

5'-O-(2,3,5,6-Tetrafluoro-4-nitrophenyl)-2'-deoxyuridine 16 (5)



16(5)

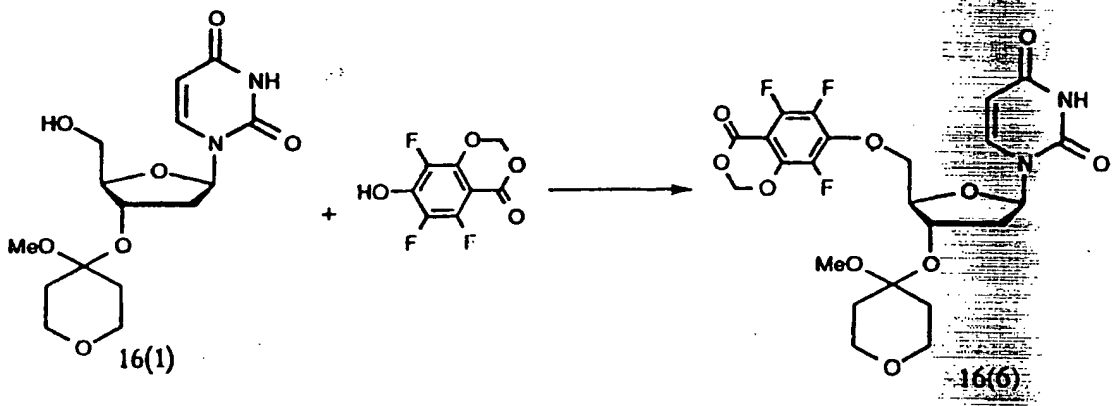
3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine 16 (2) (0.453 g, 0.85 mmol), acetic acid (4 ml), THF (2 ml), and water (1 ml) were stirred together at ambient temperature for 18 h. The resulting solution was evaporated and the residue was twice redissolved in toluene-ethanol and evaporated. Chromatography on silica (Merck no. 7729) using dichloromethane-ethanol (stepwise gradient: 100:0, 97:3, 96:4 and 95:5) as eluant, followed by crystallisation from aqueous ethanol, afforded the title compound 16 (5) as colourless needles (0.283 g, 79%), mp 157 ^{o}C.

Analysis found: C, 42.89; H, 2.66; N, 9.99; F, 18.14. $C_{15}H_{11}F_4N_3O_7$ requires: C, 42.77; H, 2.63; N, 9.97; F, 18.04%.

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 2.22 (m, 2 H, 2'-H), 4.08 (m, 1 H, 4'-H), 4.34 (m, 1 H, 3'-H), 4.65 (m, 2 H, 5'-H), 5.48 (d, J 4.4, 1 H, OH), 5.61 (d, J 8.0, 1 H, 5-H), 6.20 (t, J 6.8, 1 H, 1'-H), 7.62 (d, J 8.1, 1 H, 6-H), 11.31 (s, 1 H, N3-H). $\delta_F(CD_3SOCD_3 + FCCl_3)$ -155.05 (m, 2 F, 2,6-F) -147.13 (m, 2 F, 3,5-F).

Mass Spectrum: m/z (FAB$^+$) 444 [(M + Na)$^+$, 20%], 422 [(M + H)$^+$, 75%], 310 [(M + H - uracil)$^+$, 64%].

3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(4-oxo-5,6,8-trifluoro-4H-1,3-benzodioxin-7-yl)-2'-deoxyuridine 16 (6)



Diisopropyl azodicarboxylate (DIAD) (0.10 g, 0.5 mmol) was added dropwise during 5 min to a stirred, cooled (ice-water bath) solution of 3'-O-(4-methoxytetrahydropyran-4-yl)-2'-deoxyuridine 16 (1) (Y. Ji, W. Bannwarth and B. Luu, Tetrahedron, 1990, 46, 487) (0.157 g, 0.46 mmol), 5,6,8-trifluoro-7-hydroxy-4H-1,3-benzodioxin-4-one (compound 15 (8)) (0.096 g, 0.44 mmol), and triphenylphosphine (0.120 g, 0.46 mmol) in dry THF (3 ml) under argon. The mixture was then allowed to come to room temperature. After 2 h further triphenylphosphine (0.040 g, 0.15 mmol) and DIAD (0.033 g, 0.16 mmol) were added. After a further 1 h the mixture was concentrated and the residue partitioned between chloroform (70 ml) and half-saturated aqueous sodium hydrogen carbonate (40 ml). The aqueous layer was extracted with chloroform (10 ml) and the combined chloroform solution washed with water (20 ml), dried (Na_2SO_4) and evaporated. The residue was chromatographed on silica (Merck no. 7729) using dichloromethane-ethanol (stepwise gradient: 100 : 0, 98 : 2, 95 : 5) to give the title compound 16 (6) as a colourless glass (0.181 g, 76%).

NMR Spectra: $\delta_H(CD_3SOCD_3)$ 1.75 (m, 4 H, 3'',5''-H), 2.33 (m, 2 H, 2'-H), 3.15 (s, 3 H, Me), 3.48, 3.62 (2 x m, each 2 H, 2'',6''-H), 4.23 (m, 1 H, 4'-H), 4.59 (m, 3 H, 3'-H, 5'-H), 5.61 (d, J 8.0, 1 H, 5-H), 5.89 (s, 2 H, O-CH_2-O), 6.18 (t, J

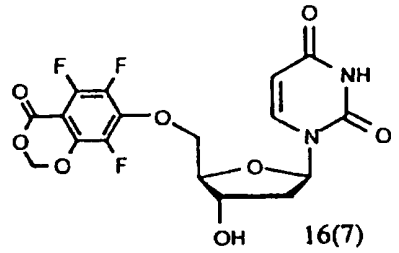
6.8, 1 H, 1'-H), 7.62 (d, J 8.0, 1 H, 6-H), 11.31 (s, 1 H, N3-H).

δ_F(CD_3SOCD_3 + $FCCl_3$) -159.20 (d, J 22.7, 1 F, 6-F), -155.09 (d, J 12.6, 1 F, 8-F), -139.40 (dd, J 11.6, 22.3, 1 F, 5-F).

Mass Spectrum: m/z (FAB$^+$) 567 [(M + Na)$^+$, 9%], 545 [(M + H)$^+$, 16%].

Measured molecular mass = 545.1370; actual molecular mass [(M + H)$^+$] = 545.1383.

5'-O-(4-Oxo-5,6,8-trifluoro-4H-1,3-benzodioxin-7-yl)-2'-deoxyuridine 16 (7)

16(6) ⟶ 

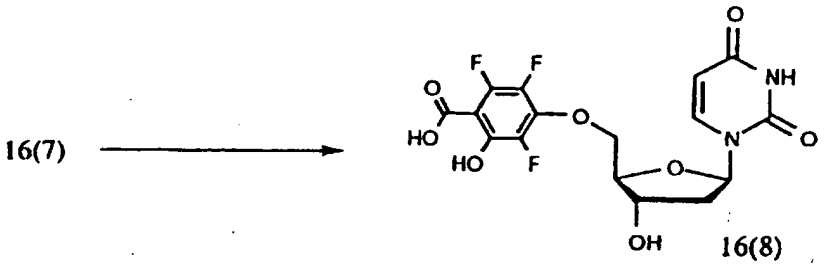
16(7)

3'-O-(4-Methoxytetrahydropyran-4-yl)-5'-O-(4-oxo-5,6,8-trifluoro-4H-1,3-benzodioxin-7-yl)-2'-deoxyuridine 16 (6) (0.081 g, 0.15 mmol), acetic acid (2 ml), and water (0.5 ml) were stirred together at room temperature for 3.5 h. The solution was evaporated and the residue was twice redissolved in ethanol-toluene and evaporated. It was then chromatographed on silica (Merck no. 7729) using dichloromethane-ethanol (stepwise gradient: 100 : 0, 98 : 2, 97 : 3, 96 : 4) as eluant. Appropriate fractions were combined and evaporated and the residue was triturated with diethyl ether, dried and crystallised from ethanol to give the title compound 16 (7) (0.023 g, 36%), mp 189-191 °C. Analysis found: C,47.34; H,3.25;N,645.$C_{17}H_{11}F_3N_2O_8$ requires: C, 47.45;H,3.05;N,6.51%.

NMR Spectra: δ_H(CD_3SOCD_3) 2.22 (m, 2 H, 2'-H), 4.07 (m, 1 H, 4'-H), 4.35 (m, 1 H, 3'-H), 4.6 (m, 2 H, 5'-H), 5.45 (d, J 4.4, 1 H, 3'-OH), 5.60 (d, J 8.1, 1 H, 5-H), 5.89 (s, 2 H, O-CH_2-O), 6.20 (t, J 6.8, 1 H, 1'-H), 7.62 (d, J 8.0, 1 H, 6-H), 11.29 (br. s, 1 H, N3-H).

δ_F(CD_3SOCD_3 + $FCCl_3$) -159.12 (d, J 22.4, 1 F, 6-F), -154.99 (d, J 11.6, 1 F, 8-F), -139.40 (dd, J 11.9, 22.0, 1 F, 5-F).

4-(2'-Deoxyuridin-5'-yloxy)-2,3,5-trifluoro-6-hydroxybenzoic acid 16 (8) (=CB 7747)



5'-O-(4-Oxo-5,6,8-trifluoro-4H-1,3-benzodioxin-7-yl)-2'-deoxyuridine 16 (7) (0.019 g, 0.044 mmol), was stirred with 0.1 M aqueous sodium hydroxide solution (1.3 ml) at room temperature for 50 min. Methanol (2.5 ml), water (2.5 ml), and acetic acid (2 drops) were added and the resulting mixture was applied to a column (bed volume 4 ml) of Biorad AG 50W-X4 100-200 mesh cation exchange resin (H^+ form). The column was eluted with methanol-water (1:1 v/v) and the eluate partly concentrated and cooled to 5 °C resulting in separation of the title compound 16 (8) as a white solid which was collected by centrifugation and dried. Yield 0.010 g, 54%; mp 228-230 °C (decomp). Analysis found: C,45.15;H,3.35;N,6.52. $C_{16}H_{13}F_3N_2O_8.0.5H_2O$ requires: C,44.98;H,3.30;N,6.56%.

NMR Spectra: δ_H(CD_3SOCD_3) 2.19 (m, 2 H, 2'-H), 4.04 (m, 1 H, 4'-H), 4.35 (m, 1 H, 3'-H), 4.46 (m, 2 H, 5'-H), 5.59 (dd, J 2.1, 8.1, 1 H, 5-H), 6.20 (t, J 6.8, 1 H, 1'-H), 7.63 (d, J 8.1, 1 H, 6-H), 11.29 (s, 1 H, N3-H).
δ_F(CD_3SOCD_3 + $FCCl_3$) -166.12 (d, J 22.6, 1 F, 3-F), -157.10 (d, J 8.6, 1 F, 5-F), -140.88 (dd, J 8.9, 22.8, 1 F, 2-F).

Mass Spectrum: m/z (ESI, -ve ion mode) 417.1 [$(M - H)^-$, 100%], 373.1 [$(M - CO_2H)^-$, 36%].

EXAMPLE 17

BIOCHEMICAL ASSAY FOR RAS PROTEIN FARNESYLTRANSFERASE (FTase) AND GERANYLGERANYLTRANSFERASE I (GGTase)

References:

Harwood, J. H. *Anal. Biochem.* 226 (1995), 268 278

Moores, S. L. *et al., J. Biol. Chem.* 226 (1991), 14603-14610.

For the FTase assay, activity was determined by the transfer of tritiated farnesyl from [1-^{3}H] farnesylpyrophosphate (FPP) to the H-ras (Wild Type - CVLS) protein. The GGTase assay involved the use of [1-^{3}H] geranylgeranylpyrophosphate as cofactor and H-ras (CVLL) as acceptor protein. With both enzymes, the tritiated labelled protein was separated from the other assay components by precipitation with acidic ethanol followed by filtration on Whatman GF/C glass fibre filter paper. Liquid scintillation counting of the filters enabled the product to be quantified. Test compounds were examined across a range of concentrations up to 100 μM and IC_{50}values determined from the log dose against response curves. Rat brain cytosol was used as a source of both the FTase and GGTase enzymes, in addition partially purified FTase enzyme (ammonium sulphate fractionation followed by ion exchange chromatography) was obtained from human placenta. For both assays, the substrate concentration for the Ras protein was 5 μM and 0.5 μM for the labelled cofactor, under these conditions, activity was linear up to 30 mins of incubation and a protein concentration of 1.25 mg/ml.

Established inhibitors α-hydroxyfarnesyl phosphonic acid and chaemomellic acid A were also tested for comparative purposes.

EXAMPLE 18

Biochemical assay for Squalene Synthase (SQS)

References:

Harwood, J. H. *Anal. Biochem.* 226 (1995), 268-278

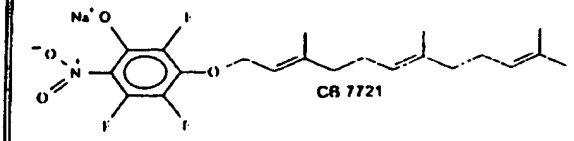
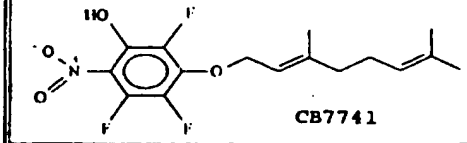
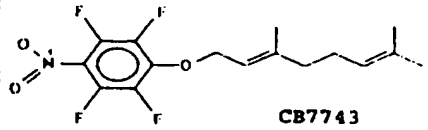
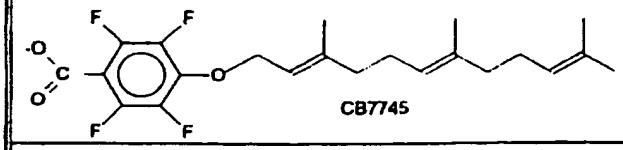
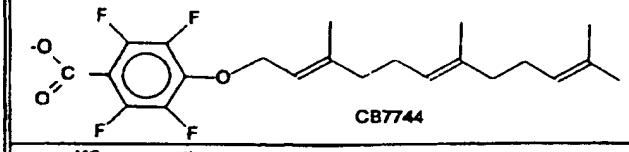
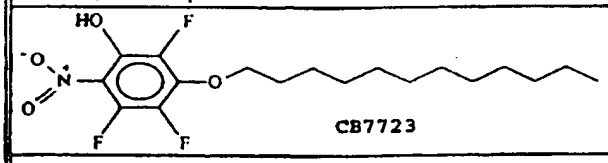
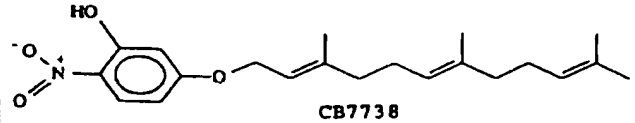
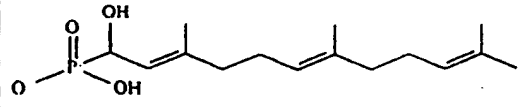
McTaggert F. *et al., Biochem. Pharmacol.* 51 (1996), 1477-1487

SQS catalyses the dimerization of farnesylpyrophosphate (FPP) to squalene via presqualene pyrophosphate. The assay

consists of incubating [^{3}H] FPP with the cofactor NADPH and rat liver microsomes under anaerobic conditions. Samples were applied to a C_{18} Bond Elut column which retains FPP, the tritiated labelled product, squalene was eluted by washing with 10% ethyl acetate in hexane and quantified by liquid scintillation counting. IC_{50} values were generated as described for the previous assays.

The results of these assays are given in the following Table:

Results of In Vitro Biochemical Assays - IC_{50} (μM)

	GGTase I Rat Brain	RAS FTase		SQS
		Rat Brain	Human Placenta	Rat Liver
 CB 7721	12.5	6.3	4.3	69
 CB7741	nd	7.5	nd	>100
 CB7743	nd	>100	nd	nd
 CB7745	nd	>100	nd	nd
 CB7744	30	>100	nd	>100
 CB7723	nd	>100	>100	91
 CB7738	nd	>100	>100	>100
 α -hydroxyfarnesylphosphonic acid	nd	2.7	0.86	17
 chaetomellic acid A	40	1.8	0.90	100

Comparison of CB7741 and CB7721 shows that farnesyl can be replaced by geranyl and still retain FTase inhibitory activity. Comparison of CB7738 (the CB7721 analogue lacking the 3 fluorines on the phenyl ring) with the corresponding fluorine substituted compound, CB7721 shows that the fluorine substitution is required for the described activity.

A further comparative compound (known as CB7723) was also tested. In this compound the farnesyl chain is replaced with an unfunctionalised chain of the same length. The result for compound CB 7723 shows that the triphosphate mimicking group alone does not confer activity when attached to an unfunctionalised chain, which confirms that the compound having the farnesyl chain exhibits true mimicry.

EXAMPLE 19

This Example demonstrates that a GDP mimic according to the invention binds to a mutant Ras protein in a competitive binding assay.

Principles of Competitive Binding Assays

A competitive binding assay system was devised, taking advantage of the protein already being saturated with nucleotide.

A nucleotide or nucleotide mimic was titrated against tritiated GTP, for a fixed number of saturated guanine nucleotide binding sites (that is a fixed concentration of protein). If the nucleotide or mimic was competing with the tritiated GTP, then a reduction in c.p.m due to the reduced ability of tritiated GTP to bind was observed at equilibrium. It has been demonstrated, that under low Mg^{2+} conditions (less than 0.5μM), both GTP and GDP dissociate rapidly from the nucleotide binding site of ras p21 proteins, with nucleotide exchange occurring in 10 minutes.

Materials

General: Dithiothreitol (DTT) was obtained from Calbiochem. Ethylenediaminetetraacetic acid (EDTA) disodium salt, and guanosine 5'-triphosphate (GTP) lithium salt, were obtained from The Sigma Company. Magnesium chloride, sodium chloride and Trizma™ were obtained from Fisons, Rose Chemicals and The Sigma Company respectively. 25mm nitrocellulose filters with 0.45μm pore size were obtained from Millipore. The GDP and GTP mimics were 5'-O-(2,3,5-trifluoro-6-hydroxy-4-pyridyl)guanosine and 5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)guanosine. All incubations were performed in 2ml Eppendorf tubes.

Buffers: Incubation buffer consisted of 20mM tris/HCl pH 7.5, 100mM NaCl, 5mM EDTA and 1mM DTT.

Wash buffer consisted of 20mM tris/HCl pH 7.5, 100mM NaCl, and 5mM $MgCl_2$.

Radioactive isotope: Tritiated GTP was obtained from Amersham International, typically, as 250μCi in a volume of 250μl of 1:1 aqueous ethanol. Isotopes were stored at -20°C, and were used within 1 month of purchase, to assure minimum exchange of the tritium label. Any isotope dilutions were performed immediately prior to experimentation and were checked for accuracy by scintillation counting. All tritiated GTP was used with a final specific activity of 6.9 Ci/mmol.

Pipettes: Electronic Dispensing Pipette (EDP) was from Ranin and other measurements were made using 20 and 100μl pipettes from Gilson.

Methods and Optimisation of Assay Conditions

Selection of Incubation Temperature: A difference of opinion exists as to which temperature is the best used to obtain optimum conditions for equilibrium formation. It has been suggested that 25°C is best, whereas others suggest 37°C. 30°C was chosen as a reasonable value.

Concentration of EDTA: It has been observed that in the presence of Mg^{2+} and GTP, ras p21 exists as a stable complex with a very slow exchange rate (half-life around 20 minutes). In very low Mg^{2+} concentrations (less than 0.5μM), the off-rate for GDP is very fast, with a half-life of less than 30 seconds. In order to obtain similar, rapid nucleotide exchange conditions in these assays, the incubation buffer contained 5mM EDTA to chelate free Mg^{2+} ions. A maximum value for the Mg^{2+} concentration was calculated to be 0.13μM using the equilibrium constatns and following equation:

$$[Mg^{2+}_{tot}] = [Mg^{2+}]\{1+[EDTA]/([Mg^{2+}]+K)\}$$

$[Mg^{2+}]$ is the concentration of free (non-chelated) Mg^{2+} in solution

$[Mg^{2+}_{tot}]$ is the initial concentration of Mg^{2+} in the assay solution

K is the equilibrium constant for EDTA binding Mg^{2+}

Duration of Incubation: A time course reaction was performed, using similar assay conditions proposed for the competitive binding studies, to evaluate the length of time necessary to reach a point of equilibrium for nucleotide exchange.

Other Conditions: NaCl and DTT concentrations were identical to those used in the final protein purification buffer.

Method for Time Course: Reaction mixtures for the time course experiment were prepared in quadruplicate at 25°C, and contained the following final concentrations: 20mM tris/HCl (pH 7.5), 100mM NaCl, 4mM EDTA, 1mM DTT, and 1mM $MgCl_2$ (final concentration of free Mg^{2+} ions was not greater than 0.13μM, see above calculation), in a total of 50μl. Protein was added last, to a concentration of 8μM. Samples were then briefly vortexed and finally centrifuged. Incubations were performed for up to 1 hr at 30°C. Sampling occurred every 10 minutes. The reaction was then quenched with 0.95ml of cold (4°C) buffer, used so that protein containing bound tritiated

nucleotide was not exposed to a change of ionic strength, which could possibly lead to loss of the nucleotide from the binding site. Bound tritiated GTP was determined by filter binding as follows:

Filters (25mm nitrocellulose, with 0.45μm pore size, from Millipore), were soaked for 20 minutes in incubation buffer prior to use, as apparently this increases the reliability of the technique. They were then placed on a Millipore multi filter unit. Quenched samples were then filtered under vacuum, using 10ml of cold incubation buffer to wash each filter. The filters were dried under a stream of hot air and placed in glass scintillation vials (Packard, 20ml). 10ml scintillation fluid (Packard, Emulsifier-safe™) was added, and the filters allowed to dissolve. Scintillation counting (Pharmacia scintillation counter, linked to a Wallac H210 personal computer) was performed using a 6 minute c.p.m counting program.

Method for Competitive Studies:

Prior to experimentation, solutions or the likely GDP and GTP mimics (see Examples 1 and 2 respectively), were prepared as follows:

The likely GDP mimic was dissolved in 50mM NaOH to a concentration of 0.5mM. From this stock solution, various dilutions were prepared using incubation buffer. These diluted solutions were checked spectroscopically, immediately after dilution and after 12 hrs, for any sign of chemical change or altered solubility.

The likely GTP mimic was dissolved in incubation buffer to a concentration of 1mM. Stocks were prepared 12 hrs before use, and were stored at 25°C. Stocks were diluted to the concentrations to be used in the binding assays and were also checked spectroscopically. This indicated that the concentrations used were accurate, that is, the compound was soluble at that concentration and suggested that no chemical alteration of the compound had occurred.

Reaction mixtures were prepared as before, in total of 50µl, with the same final chemical concentrations, except competitive conditions were used. Proteins were added to a final concentrations of 8µM. Samples were vortexed briefly and then centrifuged. Incubations were performed for 40 minutes, filter binding and scintillation counting were performed as for the time course experiment.

Results

Brief Description of the Drawings

Figure 1. Time course experiment 0.5µM tritiated GTP was incubated with 8µM highly purified, truncated rac1 protein according to the method described below. Equilibrium was reached within 10 minutes. Similar results were obtained using 8µM Leu 61 H-ras and H-ras p21.

Figure 2. Competitive binding study using Leu 61 H-ras and the GDP mimic Increasing concentrations of GTP and GDP mimic were titrated against 0.5µM tritiated GTP, using 8µM highly purified truncated, Leu 61 H-ras (see method described below). Standard deviation values for 4x repeat of each data point, for the ratios 0, 10, 20, 30, 40 and 50 to 1, GDP mimic to tritiated GTP, were ± 1.3, 1.1, 1.5, 2.2, 1.6 and 1.1 respectively. These represent the following percentage errors from the mean values, ± 6, 5, 7, 12, 10 and 7 respectively. Very similar results were obtained from a second experiment, using the same source of protein.

Figure 3. Competitive binding study using Leu 61 H-ras and the GTP mimic. Increasing concentrations of the GTP mimic were titrated against 0.5µM tritiated GTP, using 8µM highly purified, truncated Leu 61 H-ras (see method described below). Data for the GTP titration was the same as that for figure 2, with additional ratios to 250:1 GTP to 0.5µM tritiated GTP.

Figure 4. Competitive binding study using H-ras p21 and the GDP mimic. The titration was performed as before with the

GDP mimic (figure 2), against 0.5μM tritiated GTP, using 8μM of highly purified H-ras p21 (see method described below).

Figure 5. Competitive binding study using H-ras p21 and the GTP mimic. The titration was performed as before with the GTP mimic (figure 3), against 0.5μM tritiated GTP, using 8μM highly purified H-ras p21 (see method described below). Data for the GTP titration was the same as that for figure 4, with additional ratios 250:1, GTP to 0.5μM tritiated GTP.

Time Course

The time course reaction (figure 1) demonstrates that equilibrium for the exchange process, for a concentration of 0.5μM tritiated GTP and 8μM rac1, was reached within 10 minutes. Similar results were obtained using the same conditions and 8μM Leu 61 H-ras and H-ras p21. Hence all further samples in the competitive studies were incubated for 40 minutes, whilst maintaining the other assay conditions. The extra incubation time to allow equilibrium to be reached was considered necessary because of the unknown behaviour of the test mimics in the assay system.

Competitive Binding Studies

The purpose of these studies was to compare the relative binding abilities of the likely nucleotide mimics with that of GTP for the nucleotide binding site of mutant Leu 61 H-ras protein. Each protein was taken in turn and a cold chase experiment performed, where GTP was used as a competitor to tritiated GTP. Experiments were then performed under the same conditions using the likely GDP and GTP nucleotide mimics. Hence a direct comparison was then possible.

Figure 2 displays the competitive binding assay results for the likely GDP mimic compared to GTP, using 8μM Leu 61 H-ras. Using a concentration of 25μM GTP (50:1 ratio of GTP to tritiated GTP), a reduction in the mean c.p.m value to 21% of the original value (without competitor present) occurred. At

the same concentration of the likely GDP mimic there was a reduction in the mean c.p.m value to 79% of the original value. This result falls outside the extremes of experimental error.

Figure 3 displays the results for GTP compared to the likely GTP mimic, using 8μM Leu 61 H-ras. The data for GTP binding was taken from the experiment using the likely GDP mimic. Here, because the GTP mimic has the advantage of greater aqueous solubility, ratios of up to 250:1 mimic to tritiated GTP were obtained. There appears to be no indication of a reduction in the ability of tritiated GTP to bind, using any of the chosen ratios of the proposed GTP mimic. Again these results are subject to errors.

Figure 4 displays the results for a similar competitive binding study using GTP, the likely GDP mimic and 8μM H-ras p21. Figure 5 displays the data for the likely GTP mimic.

The GTP curve is very similar to the one obtained for the H-ras Leu 61 experiment. This is to be expected, since under identical experimental conditions similar binding displacement curves should be obtained. The position of equilibrium of nucleotide binding is likely to be subtly different for the two proteins, but this is undiscernible due to the experimental errors. There appears to be no indication of any reduction in tritiated GTP binding for any of the ratios of likely GDP or GTP mimics used, suggesting that the likely GDP mimic displays a selectively for Leu 61 H-ras over the wild type.

EXAMPLE 20

dUTPase Assay

dUTPase is an ubiquitous enzyme, responsible for the hydrolysis of deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) thus minimising the incorporation of uracil into DNA. This pyrophosphatase activity is measured by incubating cell lysates with radiolabelled dUTP and separating the product by

chromatography. The amount of product formed is determined by radioactive measurement of radiolabelled dUMP. The assay is based on that described by Caradonna and Adamkiewicz (J. Biol. Chem. 259, 5459-5464, (1984)) and the protocol followed was that used in Professor Caradonna's laboratory. In this later method the buffer used is a Tris-HCl (1M, pH 7.6) buffer which contains 1M DTT. Enzyme activity is calculated as follows:

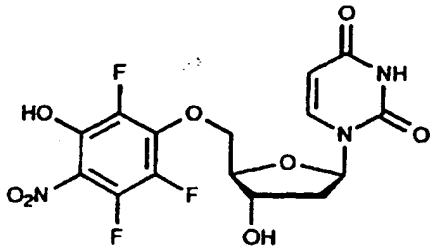
$$\frac{cpm_{extract} - cpm_{blank}}{cpm_{total}} \times \frac{0.2}{T} \times \frac{1}{P}$$

where 0.2 is nmoles dUTP in reaction, T the duration of reaction in minutes and P the amount (μg) of protein in the reaction. The units determined are nmoles/min/mg protein.

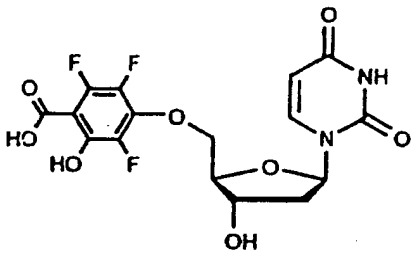
dUTP radioimmunassay

Cross-reaction of a dUTP antiserum with the dUTP phosphate mimic was determined using a radioimmunoasay (Piall EM et al. Anal. Biochem. 177, 347-352, (1989)). The assay was carried out as described in this paper except that a robotic liquid processor was used to set up the assays. Solutions of dUTP (0-20ng/ml) or dUTP mimic (0-10μg/ml) were prepared in assay buffer and incubated with radiolabelled dUTP and a limited amount of dUTP antserum (1/1000 diluiton of R9) for 2h. Antibody bound radiolabel was separated from unbound using dextran coated charcoal and the bound fraction measured using a liquid scintillation counter. Percentage binding was plotted against concentration of dUTP or dUTP mimic and the ratio of the amount of each required to inhibit binding by 50%, calculated.

Compounds CB7746 and CB7747 were evaluated using the dUTP antiserum assay.



CB 7746



CB 7747

Both compounds were supplied in 1mg aliquots. A 1mg/ml solution of each was prepared in 0.15M sodium bicarbonate solution. This was calculated to correspond to 2.31mM.

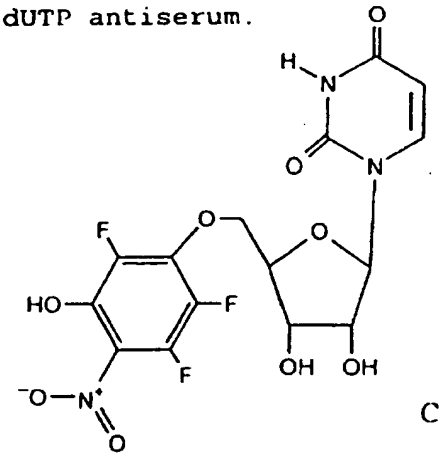
1. Cross-reaction with dUTP antiserum

The assay was carried out as described above using the Multiprobe robotic system and unpurified sheep antiserum to dUTP (R9 pool used at a dilution of 1/1000). The antiserum binding of tritiated dUTP in the presence of dUTP (20-2000fmoles) was compared to that of CB7746 and CB7747 (50fmoles-200pmoles). Figures 6 shows the cross-reaction curves obtained. Bo is the binding obtained in the absence of unlabelled dUTP and was 32.1% total tritiated dUTP.

Percentage cross-reaction was calculated at 50% displacement and was 0.6% and 2.5% for CB7746 and CB7747 respectively.

Also shown in the figure are previous results obtained with the UTP mimic CB7656, which was made using chemistry analogous to that of Example 2, which does not cross-react (<<0.06%) with the dUTP antiserum.

CB 7656



$C_{15}H_{12}F_3N_3O_9$

MW 435

5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitro)phenyluridine

The Table shows the specificity of the antiserum towards other nucleotides and nucleosides.

Compound	Percentage cross-reactivity
dUTP	100
UTP	3.0
dUMP	3.0
dUDP	42.2
TTP	2-5
dCTP	<0.076
dUrd	<2.0

The results of the examples suggest that some compounds are better at mimicking tri-phosphates than di-phosphates and vice versa. In particular, the results of the FTase assay indicate that compounds in which X is OH and Z is $=C(NO_2)-$ show greater di-phosphate mimicry than compounds in which X is OH and Z is =C(COOH)-. The results of the dUTP antiserum assay indicate that compounds in which X is OH and Z is

=C(COOH)- show greater tri-phosphate mimicry than compounds in which X is OH and Z is $=C(NO_2)-$.

Literature precedents exist for the use of diphosphate mimicking groups in the following context:

1. Inhibitors of the enzyme *purine nucleoside phosphorylase* a key enzyme in the purine salvage pathway could have utility as immunosuppressive agents, and in the treatment of T-cell leukemia, gout and some parasitic diseases as specified in S. Halazy, A.Ehrhard and C.Danzin "9-(Difluorophosphonoalkyl)-guanines as a new class of multisubstrate analogue inhibitors of purine nucleoside phosphorylase", J.Amer. Chem. Soc. 1991, 113, 325-317 and refs cited therein. In particular, based on the find cited therein that the diphosphate derivative of *acyclovir* is a potent inhibitor of the human enzyme, diphosphate mimics of the present invention replacing the diphosphate of acyclovir could be envisaged as having these therapeutic properties.

2. Ganciclovir is like acyclovir an acyclic nucleoside analogue. It is active against herpes virus and has been approved for treatment of cytomegalovirus retinitis. It acts via its conversation in vivo first into its monophosphate and ultimately into its *triphosphate* which acts as an inhibitor of viral DNA polymerase. The relevant literature background is cited in C.U. Kim, P.F.Misco, B.Y.Luh and J.C. Martin "Synthesis of a phosphonate isostere of ganciclovir monophosphate: a highly cytomegalovirus active phosphonate nucleotide analogue" Tetrahedron Letters 1990, 31, 3257-3260. Hence triposphate mimics of the present invention, replacing triphosphate in ganciclovir triphosphate, can be envisage as having ganciclovir's biological activities without the need for this in vivo transformation.

3. Adenosine triphosphate participates in a wide variety of biochemical processes. In the field of anticancer chemotherapy there is much interest in the design of inhibitors of protein tyrosine kinases, which catalyse the direct transfer of the γ phosphate group from ATP to a

tyrosine moiety (see e.g. C.H. Kruse, K.G.Holden, M.L. Pritchard, J. A. Feild, D.J. Rieman, R.G.Greig and G. Poste, "Synthesis and evaluation of multisubstrate inhibitors of an oncogene-encoded tyrosine-specific protein kinase. I" J. Med. Chem. 1988, 31, 1762-1767.

Whereas replacement of the triphosphate residue in ATP by triphosphate mimicking groups of the present invention may not lead to specific inhibition of target tyrosine kinases, it could be envisage that chemical linkage of such a triphosphate mimicking group (e.g. the hydroxytrifluorophenylcarboxylate residue through the carboxyl residue) with a tyrosine residue of tyrosine or a peptide containing it, through esterification of the phenolic hydroxyl of tyrosine, could do so in accord with concepts described, for example, in P.M. Traxler, O. Wacker, J.L. Bach, J.F. Geissler, W. Kump, T. Meyer, U.Regenass, J. L. Roesel and N. Lydon, "Sulfonylbenzoyl-nitrostyrenes: potential bisubstrate type inhibitors of the EGF-receptor tyrosine protein kinase" J. Med. Chem. 1991, 34, 2328-2337.

Also in that paper is described the advantage, with respect to potency of inhibition towards EGF-receptor tyrosine protein kinase, of incorporation into a triphosphate mimicking residue of an ortho-hydroxybenzoic acid residue which is capable of coordinating Mg++ ions as required for optimal mimicry of ATP in this reaction. Such a residue (with additional fluorine substituents) is present in one of the triphosphate mimicking groups of the present invention.

Mg++ binding is also important in biochemical reactions involving isoprenoid pyrophosphates (see D.I. Ito, S. Izumi, T. Hirata and T. Suga, "Facilitation of diphosphate group elimination from geranyl diphosphate by magnesium ion chelation in cyclic monoterpenoid synthesis" J. chem. Soc Perkin Trans I 1992, 37-39 and refs therein) and therefore an important consideration in the choice of mimicking groups in the design of inhibitors of Ras protein farnesyl transferase. Parenthetically, since biosynthesis of cholesterol also depends on the assembly of isoprenoid units, isoprenoid

pyrophosphate mimics could also have application in the treatment of hypercholesterolemia.

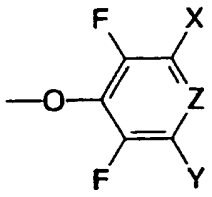
4. Mitogen-activated protein kinase (MAP kinase) has been considered a potential target for anticancer drugs. ATP and UTP have been shown to stimulate the MAP kinase cascade, so mimics of these may be potential inhibitors. Also, stimulation of MAP kinase has been implicated in mesangial cell proliferation (A. Huwiller and J. Pfeilschifter, "Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells" Brit. J. Pharmacol. 1994, 113 1455-1463), an indicator of progressive glomerular disease so such analogues have potential utility for this indication.

5. As mentioned previously, mimicry of ubiquitously utilised nucleoside di-and triphosphates may not lead to specific inhibition of individual enzymes utilising them. Nevertheless the fact that a guanosine diphosphate mimic incorporating a diphosphate mimicking groups of the invention was able to antagonise the binding of GTP of truncated leu 61 mutant form of the Ras protein but not to the normal form indicates that target selectivity can be achieved with such mimics. Such specificity may depend on the precise conformation in which the nucleotide binds in a given instance, and whether or not the mimic can simulate that particular conformation.

For example in cancer cells inhibited with thymidylate synthase inhibitors it is hypothesised that dUTP, which accumulates as a result of the TS blockade is misincorporated into DNA, promoting DNA damage and cell death. However such cells can "rescue" themselves by degrading the dUTP using dUTPase. Addition of a dUTP mimic which would be recognised by the dUTPase (and hence inhibit it) but not by the nucleoside triphosphate polymerase could potentiate the action of the TS inhibitor.

CLAIMS

1. An analogue of a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound, wherein said analogue comprises a di- or tri- phosphate mimic at the site of phosphorylation, said mimic being a moiety of the formula (I):



(I)

wherein X and Y, which may be the same or different, are fluorine or hydroxy, and Z represents an electron with drawing group or a pharmacologically acceptable salt thereof.

2. An analogue according to claim 1 wherein X is hydroxy and Y is fluoro.

3. An analogue according to claim 1 or 2 wherein Z represents either =N- or =C(R)- wherein R is carboxy or a mimic thereof, trifluoromethyl, nitro, cyano, alkyl- or aryl-sulphoxyl ($-SO_2R$), a sulphonic acid ($-SO_3H$) or ester thereof or methylene phosphonic acid ($-CH_2PO_3^{2-}$) or ester thereof.

4. An analogue according to claim 3 wherein R is a carboxy mimic which is 5-tetrazolyl.

5. An analogue according to claim 3 wherein Z represents =C(R)- wherein R is carboxy.

6. An analogue according to claim 3 wherein Z is =N- or $=C(NO_2)-$.

7. An analogue according to any one of the preceding claims wherein said biologically active compound is GTP or GDP.

8. An analogue according to any one of claims 1 to 6 wherein said biologically active compound is a farnesyl pyrophosphate.

9. An analogue according to any one of claims 1 to 6 wherein said biologically active compound is a geranyl pyrophosphate.

10. An analogue according to any one of claims 1 to 6 wherein said biologically active compound is a phosphate of deoxyuridine.

11. An analogue of a biologically active compound selected from the group consisting of:

5'-O-(2,3,5-trifluoro-6-hydroxy-4-pyridyl)guanosine;
5'-O-(2,3,5,6-tetrafluoro-4-pyridyl)guanosine;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)guanosine;
5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)guanosine;
5'-O-(3,5-difluoro-2,6-dihydroxy-4-pyridyl)guanosine;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-cyano)phenylguanosine;
5'-O-(2,3,5,6-tetrafluoro-4-carboxy)phenylguanosine;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]pyridine;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene;
2,3,5,6-tetrafluoro-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]cyanobenzene;
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]pyridine;
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene; and
2,3,5-trifluoro-6-hydroxy-4-[(E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-oxy]nitrobenzene sodium salt.

2,3,5-trifluoro-6-hydroxy-4-(trans, trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-yloxy) benzoic acid, sodium salt;
2,3,5,6-tetrafluoro-4-(trans,trans-3,7,11-trimethyl-2,6,10-dodecatrienyl-1-yloxy) benzoic acid, sodium salt;
1-[(E)-3,7-dimethyl-2,6-octadien-1-yloxy]-2,3,5,6-tetrafluoro-4-nitrobenzene;
1-[(E)-3,7-dimethyl-2,6-octadien-1-yloxy]-2,3,6-trifluoro-5-hydroxy-4-nitrobenzene;
5'-O-(2,3,6-trifluoro-5-hydroxy-4-nitrophenyl)-2'-deoxyuridine;
5'-O-(2,3,5,6-tetrafluoro-4-nitrophenyl)-2'-deoxyuridine; and
4-(2'deoxyuridin-5'-yloxy)-2,3,5-trifluoro-6-hydroxybenzoic acid.

12. A pharmaceutical composition comprising an analogue of a biologically active compound according to any one of claims 1 to 11 together with a pharmaceutically acceptable carrier of diluent.

13. An analogue of a biologically active compound according to any one of claims 1 to 11 for use in a method of treatment by therapy of the human or animal body.

14. A method of treating a disease of the human or animal body which is caused or exacerbated by a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound, which method comprises administering to a patient suffering from said disease an effective amount of a compound according to claim 1.

15. A chemical moiety of the formula (I) as defined in any one of claims 1 to 6 or a pharmacologically acceptable salt thereof, for use as a di- or tri- phosphate mimic.

16. Use of a chemical moiety of the formula (I) as defined in any one of claims 1 to 6 or a pharmacologically

acceptable salt thereof, as a di- or tri- phosphate mimic.

17. The use of an analogue according to any one of claims 1 to 11 for the manufacture of a medicament for treating a disease which is caused or exacerbated by a biologically active compound whose activity within a cell is regulated by phosphorylation of said compound.

Fig.1.

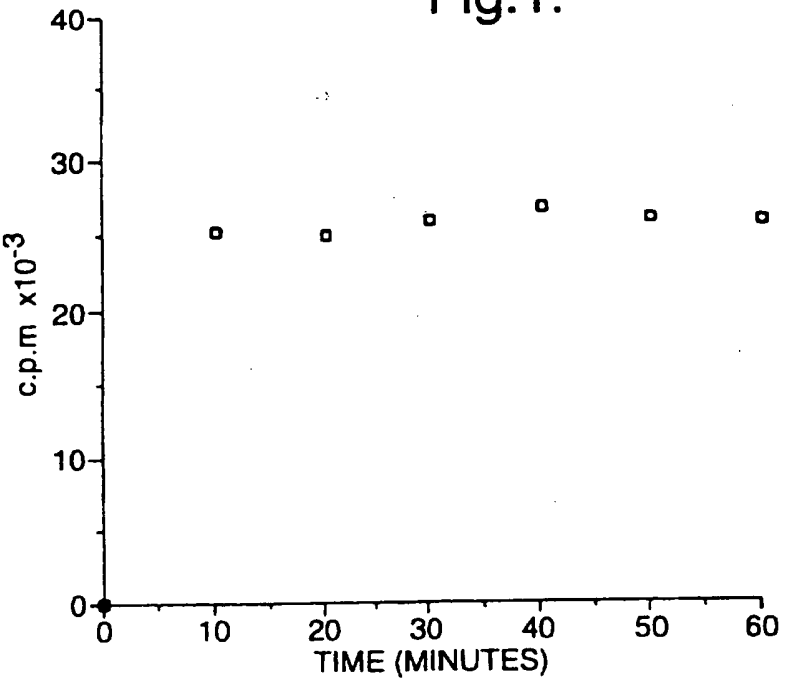

40
30
20
10
0
c.p.m x10-3
0
10
20
30
40
50
60
TIME (MINUTES)

Fig.2.

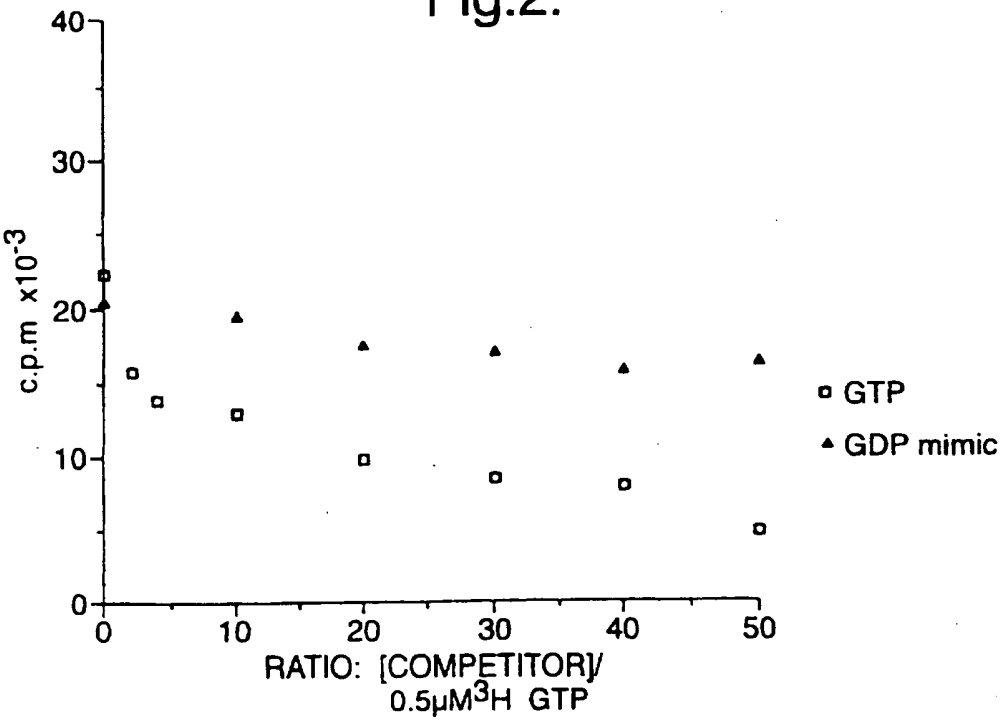

40
30
20
10
0
c.p.m x10-3
0
10
20
30
40
50
RATIO: [COMPETITOR]/
0.5μM3H GTP
GTP
GDP mimic

Fig.3.

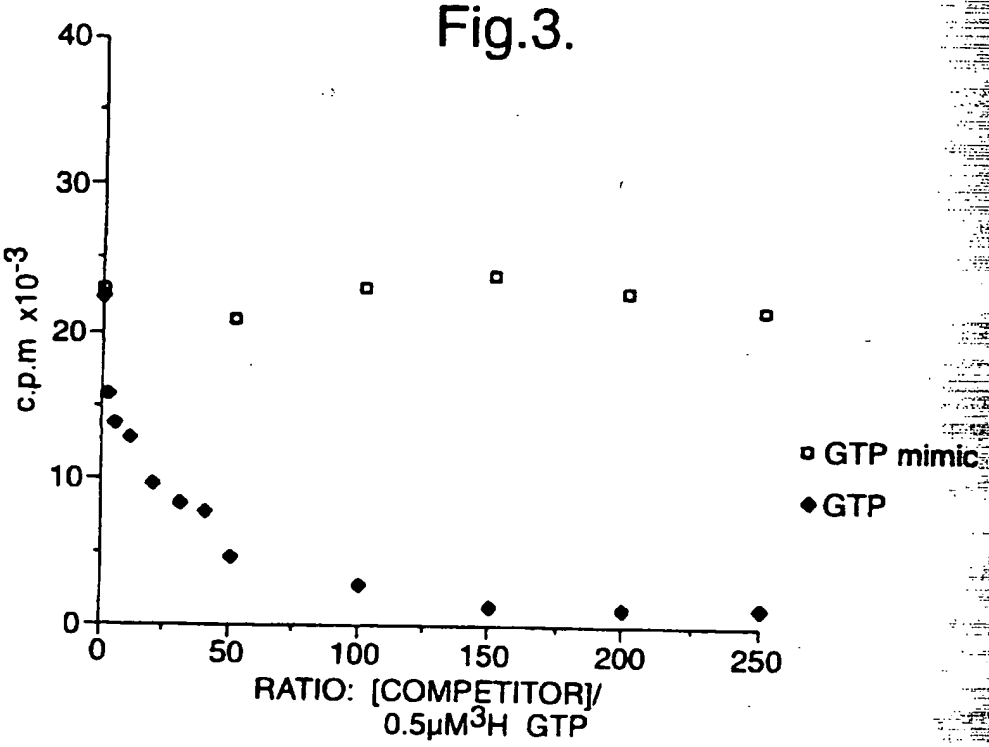

40
30
20
10
0
c.p.m x10-3
0
50
100
150
200
250
RATIO: [COMPETITOR]/
0.5μM3H GTP
GTP mimic
GTP

Fig.4.

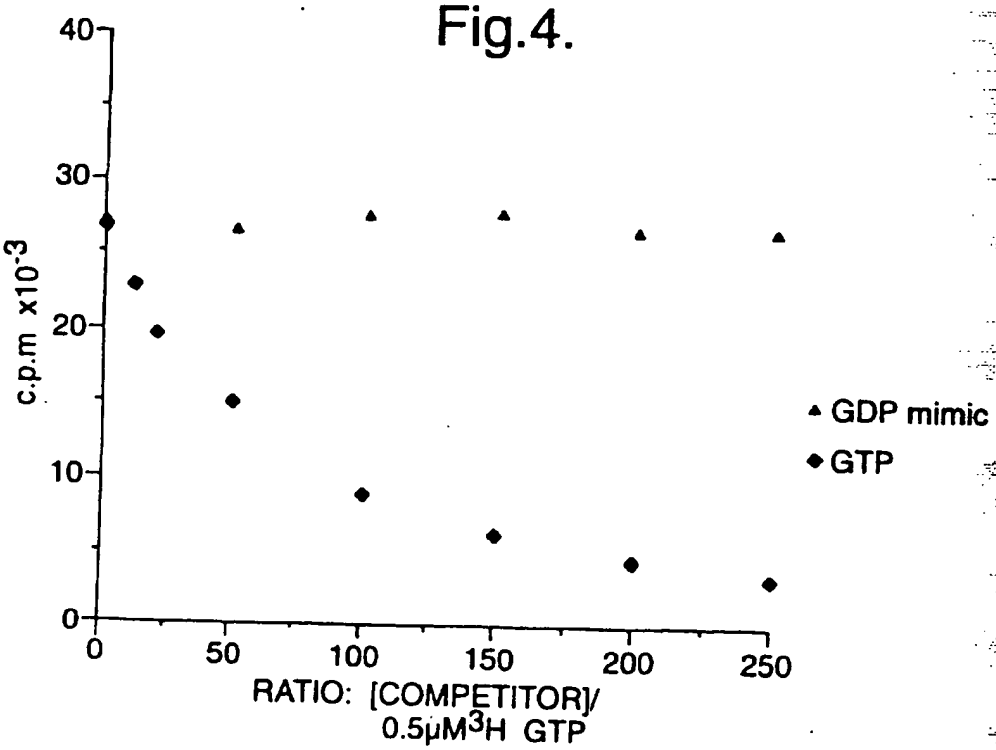
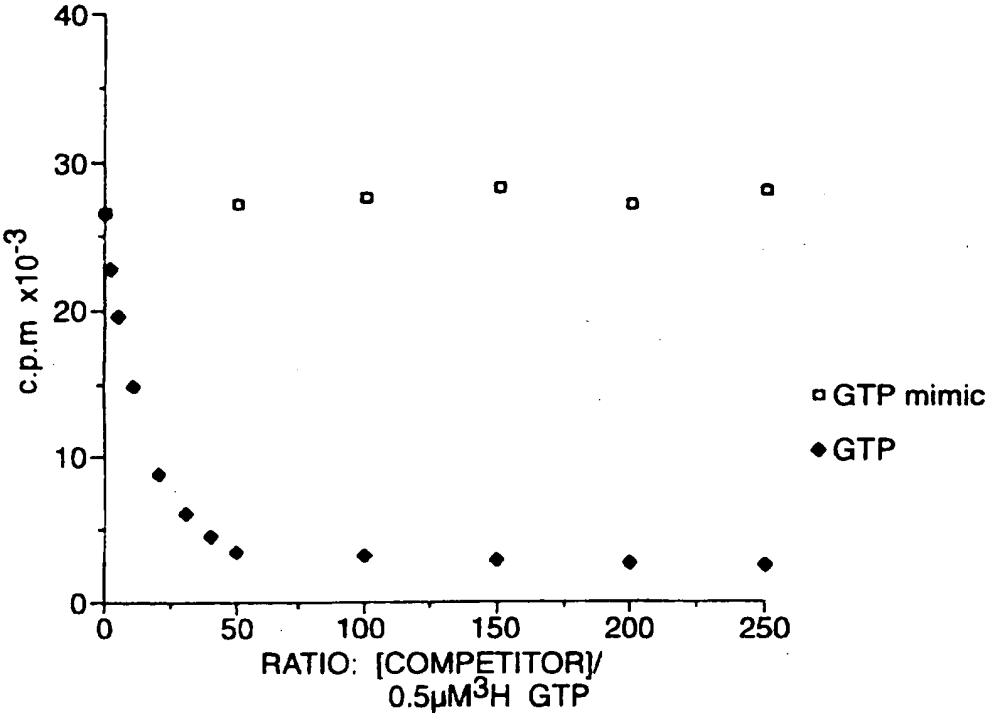

40
30
20
10
0
c.p.m x10-3
0
50
100
150
200
250
RATIO: [COMPETITOR]/
0.5μM3H GTP
GDP mimic
GTP

Fig.5.


40
30
20
10
0
c.p.m x10-3
0
50
100
150
200
250
RATIO: [COMPETITOR]/
0.5μM3H GTP
GTP mimic
GTP